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THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF SELENIUM-CONTAINING BIOLOGICAL COMPOUNDS BY NMR SPECTROSCOPY

by

KHOON SIN TAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

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EDMONTON, ALBERTA

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submitted by KHOON SIN TAN

in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Supervisor

External Examiner

DATE 20th JUNE 1986

ABSTRACT

Certain reactions of selenium-containing compounds which can be considered as models for reactions of selenium in biological systems have been studied by pulse/Fourier Transform NMR spectroscopy. This measurement technique can provide detailed information about chemical species at the molecular level. First, the complexation behaviour of methylmercury ($CH_3Hg(II)$) with several selenohydryl ligands is characterized by ^1H NMR spectroscopy. Data to calculate ligand acid dissociation constants and formation constants of the $CH_3Hg(II)$ complexes were obtained for 2-selenoethanoic acid, 3-selenopropanoic acid, 4-selenobutanoic acid, 2-hydroxyethaneselenol, selenocysteamine, selenocysteine, and selenopenicillamine. The determination of the formation constants was effected by allowing the selenohydryl ligands to compete with mercaptoacetic acid for $CH_3Hg(II)$ and monitoring the extent of the competition reaction from chemical shift data over a wide pH range. A quantitative comparison is made of the relative binding strengths of $CH_3Hg(II)$ with selenohydryl and sulphydryl ligands.

Reactions of organoselenium (e.g. selenoamino acids) and inorganic selenium (e.g. selenite) compounds with thiols were also studied. The suitability of using NMR spectroscopy as a measurement technique was investigated

by making measurements on a class of stable selenium-containing compounds, the diselenides (RSeSeR). The conditions under which sharp resonances can be observed by both ^{1}H and ^{77}Se NMR are first established. The accuracy of employing natural abundance ^{77}Se NMR spectroscopy to probe the solution chemistry of the selenium-containing compounds is evaluated.

Using the conditions thus determined, the reaction of diselenides with thiols and selenols with disulphides are

$$RSH + R'SeSeR' \rightleftarrows RSSeR' + R'SeH$$

$$RSH + RSSeR' \rightleftarrows RSSR + R'SeH$$

investigated. The characteristics of the mixed S-Se compounds, the selenenyl sulphides, have not been studied before. In particular, the acid-base chemistry of the compounds, where R and R' are alkyl groups, are studied here. Chemical shift-pH data from ^{1}H and ^{77}Se NMR spectroscopy are obtained, from which acid dissociation constants can be calculated.

The reaction of selenite with thiols is thought to be a major pathway for the incorporation of selenium into biological systems. This reaction proceeds very rapidly and the possibility of using ^{1}H NMR spectroscopy to

characterize this reaction was investigated. ^{1}H NMR measurements on the reaction of selenite with cysteamine, 2-mercaptopropanoic acid, D-penicillamine and glutathione are made. The equation describing this reaction is

$$4RSH + H_2SeO_3 \rightarrow RSSeSR + RSSR + 3H_2O$$

As there has been little documented on the solution chemistry of the selenium-containing product, the bis(alkylthio)selenide (RSSeSR), the capabilities afforded by NMR spectroscopy are exploited to characterize the acid-base behaviour of these compounds. The ^{77}Se chemical shifts reported in Chapter 5 are the first to be reported for symmetrical (RSSeSR) and asymmetrical (RSSeSR') bis(alkylthio)selenides. A comparison of the acid dissociation constants for bis(D-penicillamine)selenide calculated from ^{1}H and ^{77}Se chemical shift-pH data is also given.

The results presented in this thesis show that NMR spectroscopy can be very favourably used for characterizing the acid-base behaviour of compounds, without the necessity of having pure samples. ^{77}Se NMR data show that ^{77}Se NMR spectroscopy is a feasible, sensitive and non-invasive technique which can be used for probing the chemistry of biological selenium-containing compounds.

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D: a solution obtained from the reaction of 0.099 M D-penicillamine and 0.088 M D-,L-cysteine with 0.05 M selenite..... 226

61. 68.68 MHz ^{77}Se NMR spectra obtained during the titration of the bis(alkylthio)-selenides formed from the reaction of 0.099 M glutathione and 0.097 M cysteamine with 0.050 M selenite..... 229

FIGURE PAGE

62. The pH* dependence of the chemical shift of the ^{77}Se resonances of bis(glutathione)selenide (GSSeSG), bis(cysteamine)-selenide (CSSeSC) and the asymmetrical molecule, GSSeSC..... 230

63. NMR titration curves for a solution prepared by reacting 0.099 M D-penicillamine with 0.025 M Na_2SeO_3. A: ^{77}Se chemical shift and B: ^{1}H methine proton chemical shift of bis(D-penicillamine)-selenide is plotted as a function of pH*..... 241

CHAPTER 1

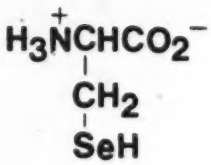
INTRODUCTION

A. Biological Occurrences and Some Reactions of Selenium-Containing Compounds

Selenium is a paradoxical element, from a biological perspective. Prior to 1957, selenium was only considered to be a toxic element, which it is at "high" concentrations (1-5). However, in 1957 it was also shown to be a nutritionally essential trace element in many species (6-8, 15-16). A fine line separates the levels which satisfy the essential nutritional requirements and the levels at which it becomes toxic. In 1980, the U.S. National Research Council proposed a safe and adequate selenium intake of 50 μg to 200 μg per day for adult humans (17). The level at which it becomes toxic is ~2400-3000 μg per day (18), i.e., only about 15 times the largest recommended daily intake to meet nutritional requirements.

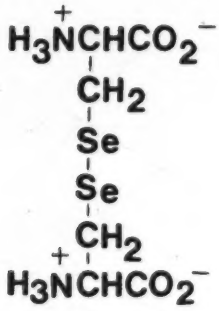
Selenium is found as a constituent of some amino acids and proteins. In these biological molecules it is generally present as a selenol (RSeH), a diselenide (RSeSeR) or a selenoether (RSeR'). The simplest

biological molecules containing the selenol, diselenide, and selenoether groups are selenocysteine [I], selenocystine [II], and selenomethionine [III], respectively. The selenium in selenoenzymes is generally present in selenocysteine residues (27,38-40).



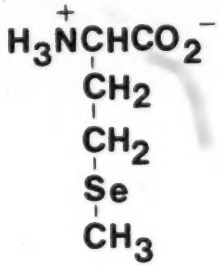
[I]

Selenocysteine



[II]

Selenocystine



[III]

Selenomethionine

The essential reactions in which the selenol, diselenide, and selenoether groups are involved in biological systems are not clearly defined. The similarities in the physical and chemical properties of sulphur and selenium leads to the expectation that their chemistry in biological systems would be similar, and this has been found to be the case for some molecules. For example, Mautner and Gunther (20) found that mole by mole replacement of sulphur by selenium in the pantethine

precursor of coenzyme A, which is necessary for the growth of Lactobaccillus helveticus, gives a molecule which is as active as the sulphur-containing cofactor. However, this is not always the case, as exemplified by studies on Escherichia coli (21). The β-galactosidase enzyme found in organisms grown in 0.01 M selenite have 8-10% of the sulphur content of this enzyme replaced by selenium and its specific activity was <50% that of the sulphur enzyme.

An important difference between the chemistry of sulphur and selenium compounds is in the greater acidity of the selenohydryl group (RSeH) as compared to that of the sulphydryl group (RSH). For instance, the pK_A of the selenohydryl proton of selenocysteine has been reported to be 5.24 (164), while the pK_A of the sulphydryl proton of cysteine is 8.39 (19). Thus, at neutral pH, the selenohydryl group is largely dissociated but not the sulphydryl group.

Low molecular weight selenium-containing compounds which have been identified in biological systems are listed in Table 1 and naturally-occurring selenoproteins are summarized in Table 2. Several other bacterial transfer ribonucleic acids have also been shown to contain selenium (48-53). Most of the selenoenzymes listed in Table 2 have a selenocysteine residue at the active site which is involved in the catalysis of redox reactions.

Table 1. Low molecular weight selenium compounds which have been identified in biological systems.

Compound	Chemical Formula	Reference
Selenocysteine	$HSeCH_2CH(NH_3^+)CO_2^-$	26,27,38
Selenocystine	$^-O_2CCH(NH_3^+)CH_2SeSeCH_2CH(NH_3^+)CO_2^-$	23
Selenohomocystine	$^-O_2CCH(NH_3^+)CH_2CH_2SeSeCH_2CH_2CH(NH_3^+)CO_2^-$	33
Se-methylselenocysteine	$CH_3SeCH_2CH(NH_3^+)CO_2^-$	24
Selenocystathionine	$^-O_2CCH(NH_3^+)CH_2SeCH_2CH_2CH(NH_3^+)CO_2^-$	22,23,28,29
Selenomethionine	$CH_3SeCH_2CH_2CH(NH_3^+)CO_2^-$	31,32
Se-methyl selenomethionine	$(CH_3)_2\overset{+}{S}eCH_2CH_2CH(NH_3^+)CO_2^-$	30
Selenotaurine	$H_2NCH_2CH_2SeO_3H$	86
Dimethyl selenide	$(CH_3)_2Se$	34,35,36
Dimethyldiselenide	$(CH_3Se)_2$	34,35,37
Trimethylselenonium ion	$(CH_3)_3Se^+$	25

Table 2. Naturally occurring selenoproteins.

Protein	Occurrence	Selenium Form	Ref
Formate dehydrogenase	E. coli & several anaerobic bacteria	Selenocysteine	39
Glycine reductase	Certain Clostridia	Selenocysteine	27
Hydrogenase	Methanococcus vannielli	Selenocysteine	40
Glutathione peroxidase	Mammals & birds	Selenocysteine	38
Nicotinic acid hydroxylase	Clostridium barkeri	*	41
Xanthine dehydrogenase	Certain Clostridia	*	42
β-hydroxylbutyrl-CoA dehydrogenase	Clostridium kluyveri	Selenomethionine	54
Thiolase	Clostridium kluyveri	Selenomethionine	32,43
80,000 M_r P1	Rat kidney & liver	Selenocysteine	44
10,000 M_r muscle protein	Bovine & ovine heart & semi tendinosus muscle	*	45
15,000 M_r protein of spermatozoa	Rat testis	*	46
^{75}Se-P protein	Rat liver	*	47

*Not known.

The selenoenzyme which has been most extensively studied and bears certain significance to the work described in this thesis is the mammalian and avian enzyme, glutathione peroxidase.

Glutathione peroxidase (GSH-Px) was discovered in 1957. However, it was not known to be a selenoprotein until 1971 (55-56). Since then, it has been extensively studied and is the subject of several recent reviews (57-59). GSH-Px (M_r = 84000) is an intracellular enzyme present in all animal tissues. It catalyzes the reduction of hydroperoxides to alcohols, using glutathione (GSH) as the reducing equivalent.

The crystal structure of GSH-Px from bovine erythrocytes has been obtained by X-ray crystallography (60). It is a tetrameric enzyme containing one selenium atom per subunit; in the reduced form of the enzyme, the selenium atoms are present as selenocysteine residues (61-63). The amino acid sequence in the region of the active site selenocysteine of GSH-Px from rat liver has recently been published (64). During the catalysis of the reduction of hydroperoxides by glutathione, the active site selenium cycles between reduced and oxidized forms. The nature of the active site in the oxidized form of GSH-Px has not been established; at least three different forms have been suggested (65-66). X-ray photoelectron

spectroscopic (ESCA) studies indicate that an oxygen atom is bound to selenium in the oxidized form of the enzyme (67). Other experiments, also by X-ray photoelectron spectroscopy (68), suggest a Se-S unit in the oxidized form. These inconsistent observations have been explained as being due to differences in the methods of handling and treatment of the enzyme.

Several mechanisms have been proposed for the catalytic reduction of hydroperoxides by glutathione peroxidase, including the simple mechanism given by equations 1-3:

$$\text{GSH-Px-SeH} + \text{ROOH} \rightarrow \text{GSH-Px-SeOH} + \text{ROH} \qquad [1]$$

$$\text{GSH-Px-SeOH} + \text{GSH} \rightarrow \text{GSH-Px-SeSG} + H_2O \qquad [2]$$

$$\text{GSH-Px-SeSG} + \text{GSH} \rightarrow \text{GSH-Px-SeH} + \text{GSSG} \qquad [3]$$

The properties of GSH-Px have been studied in detail, including its suitability as an enzyme for the protection of cells against oxidative damage by hydroperoxides (69) and its inhibition by mercaptocarboxylic acids and mercaptans (70), gold(I) antirheumatic drugs (71-73), such as gold(I) thioglucose and gold(I) thiomalate, and metal ions, such as Cd(II), Hg(II), and Pb(II) (74).

Dietary selenium can be in either an inorganic, e.g.

selenite and selenate, or an organic, e.g. the selenoamino acids, form. The incorporation of selenium into various organisms has been the subject of a large volume of research; however, the pathways by which it is incorporated have not been elucidated at the molecular level. The selenium in selenite and selenate has been shown to be readily incorporated into various amino acids in plants (23-24). It is seen from Table 2 that many of the selenoenzymes contain selenocysteine. This has led to studies on the biosynthesis of selenocysteine. Pathways proposed for the mammalian metabolism of inorganic selenium are summarized in Figure 1 (75).

B. This Dissertation

The subject of this dissertation is an investigation of certain reactions of selenium-containing compounds which can be considered as models for reactions of selenium in biological systems. The principal technique used in this work is nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy was chosen as the measurement technique because it can provide detailed information at the molecular level. Both proton (^{1}H) and selenium (^{77}Se) NMR have been used in this research. A major objective has been to evaluate the utility of ^{77}Se

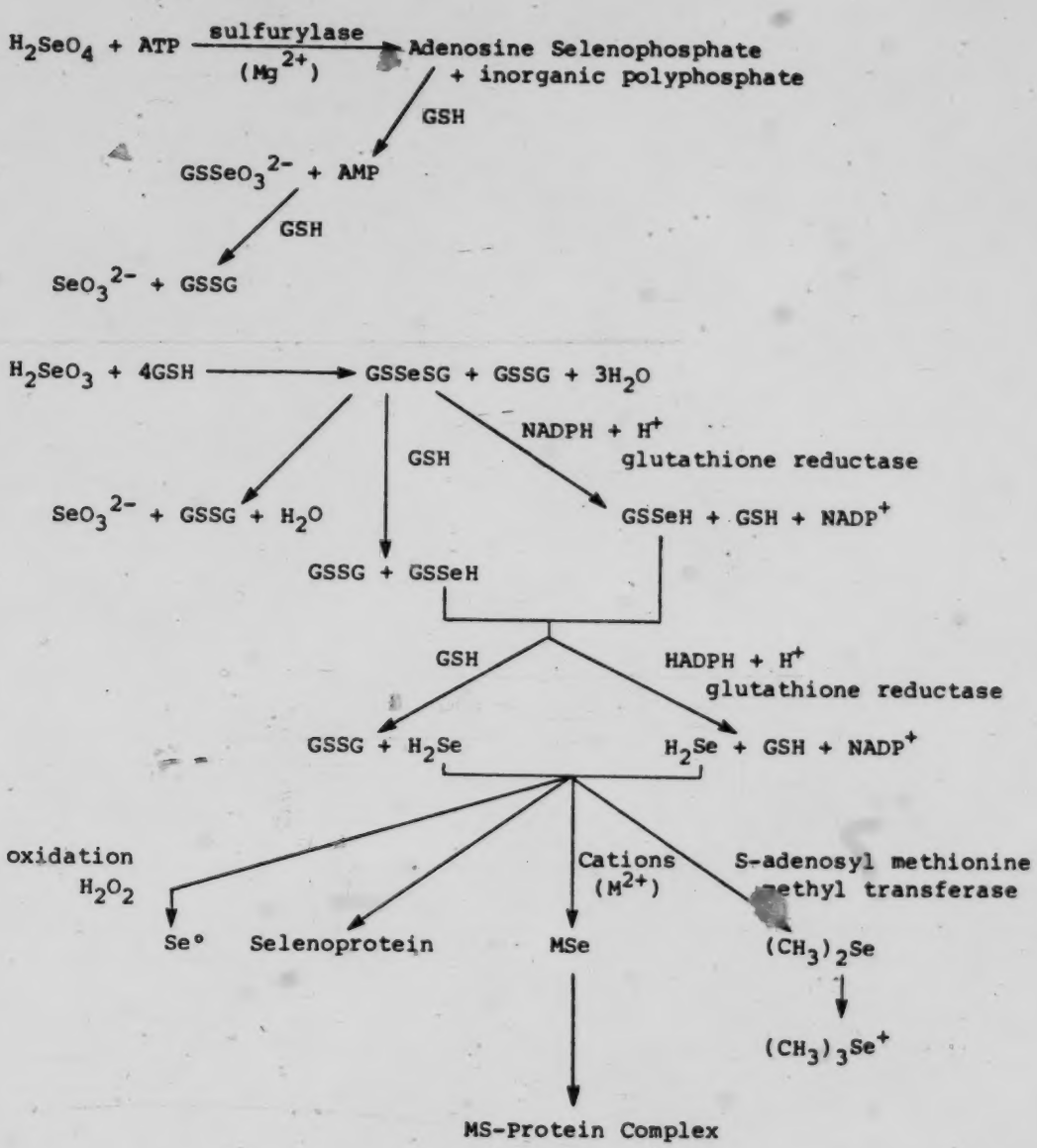


Figure 1. Mammalian metabolism of inorganic selenium (ref. 75).

NMR as a probe of the chemistry of selenium in biological molecules.

1. ^{77}Se NMR Spectroscopy

The ^{77}Se isotope has a spin 1/2 nucleus and occurs with a natural abundance of 7.58%. Natural abundance ^{77}Se NMR has a sensitivity of 5.30×10^{-4} relative to ^{1}H NMR (92). For comparison, natural abundance ^{13}C NMR has a sensitivity of 1.76×10^{-4} relative to ^{1}H NMR. The early studies on ^{77}Se NMR were by continuous wave techniques which, because of their low signal-to-noise ratio, required the use of neat liquid samples. ^{77}Se NMR parameters were also determined in some early studies by double resonance experiments (^{1}H observe, ^{77}Se decouple); because of the greater sensitivity of ^{1}H NMR, less concentrated solutions could be used. The first reported use of the pulse/Fourier transform NMR technique to do ^{77}Se NMR was by Gronowitz (98). The development of pulse/FT NMR has greatly facilitated the observation of relatively insensitive nuclei, including the ^{77}Se nucleus (93).

Most ^{77}Se NMR measurements have been made on organo-selenium compounds with low molecular weights. An important finding in these measurements is that there is no detectable enhancement of the intensity of ^{77}Se

resonances by the nuclear Overhauser effect (94-96), which indicates the absence of contributions from ^{1}H-^{77}Se dipole-dipole interactions to the ^{77}Se spin-lattice relaxation mechanism. Spin-lattice relaxation has been found to be predominantly by the chemical shift anisotropy mechanism at magnetic field strengths greater than 5 Tesla (97). Selected T_1 values are listed in Table 3. The mechanism by which ^{77}Se undergoes spin-spin relaxation does not appear to have been studied.

Chemical shift data for ^{77}Se in a large number of compounds have been reported in the literature. The chemical shift range for ^{77}Se NMR is greater than 3000 ppm. Representative chemical shift data for ^{77}Se in organoselenium and metal-organoselenium compounds are presented in Table 4.

Compounds which have been used as ^{77}Se chemical shift references include dimethylselenide, $(CH_3)_2Se$ (104); selenophene, (98,105); 4,4-dimethyldiphenyl diselenide, $(4\text{-}CH_3C_6H_4Se)_2$ (106); seleninyl chloride, $SeOCl_2$ (107), and selenious acid, H_2SeO_3 (108). Of these compounds, dimethylselenide has been studied in most detail with respect to the dependence of its chemical shift on concentration, solvent and temperature, and thus it is generally accepted as the ^{77}Se NMR chemical shift reference for ^{77}Se NMR.

Table 3. ^{77}Se spin lattice relaxation times for selected selenium compounds.

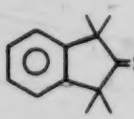
Compound	Conditions	T_1(s)	^{77}Se Frequency (MHz)	Ref
$(CH_3)_2Se$	$CDCl_3$, 32°C	7.5	19.1	96
$(CH_3Se)_2$	0.5 M $CDCl_3$, 45°C	9	19.1	96
CH_3SeH	Acetone-d_6, 40°C	1.3	19.1	96
H_2SeO_3	0.5 M D_2O, pH 9.8, 10°C	2.8	19.1	96
$Zn(Se_2CN(C_2H_5)_2)_2$	0.53 M $CHCl_3$, 27°C	4.5	19.1	99
$HSeCH_2CH_2NH_2$	0.5 M D_2O, pD 8.3, 32°C	7.1	19.1	96
D-L-$CH_3SeCH_2CH_2CHCO_2H$ (with NH_2)	0.1 M D_2O, pD 4, 34°C	13.5	19.1	96
$(C_6H_5Se)_2$	0.5 M $CDCl_3$, 45°C	20.0	19.1	97
		12.5	57.2	97
	0.5 M $CDCl_3$, 25°C	1.12	19.1	97
		0.18	57.2	97
$(CH_3)_3SeI$	1.0 M D_2O, pD 7, 32°C	13.7	19.1	96
$CH_3SeCH_2CH_2NH_2$	0.5 M $CDCl_3$, 43°C	8.4	19.1	96

Table 4. ^{77}Se chemical shifts for selected compounds.

Compound	Conditions	Chemical Shift[a] (ppm)	Ref
H_2Se	1 M D_2O	-288	107
H_2SeO_3	sat. H_2O	1282	109
Na_2SeO_3	sat. H_2O	1253	110
K_2SeO_4	sat. H_2O	1024	110
$SeOCl_2$	liquid	1479	107
KSeCN	~0.5 M EtOH, 30°C	-322	99
CSe_2	~15% w/v CH_2Cl_2	299	99
$(SiH_3)_2Se$	liquid	-666	111
$(CH_3)_2Se$	liquid, 32°C	0	103
$(C_6H_5)_2Se$	liquid	402	112
CH_3SeH	liquid	-116	103
C_6H_5SeH	liquid	152	103
$(CH_3)_2Se_2$	0.5 M $CDCl_3$, 45°C	281	96
$(CH_3)_2Se_2Cl_2$	CH_2Cl_2	448	103
CH_3SeCN	$CDCl_3$	125	113
$(C_6H_5)_2Se_2$	H_2O	460	112
$C_6H_5CH_2SeCH_2CO_2H$	$(CD_3)_2CO$	288	105
p-$FC_6H_4SeCH_3$	liquid	233	114
$((CH_3)_3C)_2CSe$	$CDCl_3$	2131	115
CF_3SeH	?	287	116

(CONTINUED)

Table 4. (Continued)

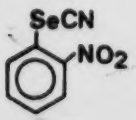
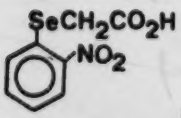
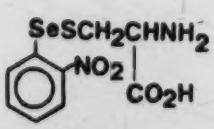
Compound	Conditions	Chemical Shift[a] (ppm)	Ref
	$CDCl_3$	605	113
$(CH_3)_3PSe$	CH_2Cl_2	-235	117
$Cd(Se_2CNC_4H_9)_2$	0.15 M $CDCl_3$, 29°C	717	99
$Zn(Se_2CN(C_2H_4)_2)_2$	0.12 M $CDCl_3$, 29°C	656	99
$Ni(Se_2CN(C_2H_5)_2)_2$	sat. $CDCl_3$, 32°C	374	99
$Pt(Se_2CN(C_2H_5)_2)P(C_6H_5)_3CH_3$	0.15 M $CDCl_3$, 33°C	387	99
$Pd(Se_2CN\text{-}(iso\text{-}C_4H_9)_2)_2$	0.16 M $CDCl_3$, 30°C	366	99
Seleno-D-,L-cystine	0.27 M D_2O, 30°C	293	99
Seleno-D-,L-methionine	0.1 M D_2O, pD 4, 34°C	75	96
$HSeCH_2CH_2NH_2$	0.5 M D_2O, pD 8.3, 32°C	-212	96
D-,L-$HSeCH_2CHCO_2H$ (with NH_2)	0.1 M D_2O, pD 5	-141	96
$C_6H_5SeSC_6H_5$	10% v/v $CDCl_3$, 20°C	526	101
	10% v/v $CDCl_3$, 20°C	738	101
	10% v/v $CDCl_3$, 20°C	610	101
	10% v/v $CDCl_3$, 20°C	574	101

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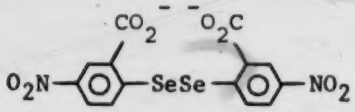
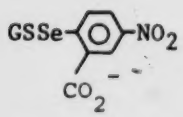
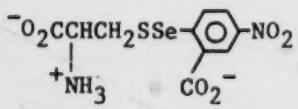
Table 4. (Continued)

Compound	Conditions	Chemical Shift[a] (ppm)	Ref
	0.14 mM, pH 8.2, 20°C	474	100
	D_2O, 20°C	576	100
	D_2O, 20°C	573	100
$(CH_3)_3SeI$	1.0 M D_2O, pD 7, 32°C	258	96

[a]Relative to $(CH_3)_2Se$

Although ^{77}Se chemical shift data have been reported for a large number of organoselenium and metal-organoselenium compounds there have been relatively few reports of the use of ^{77}Se NMR to study the chemistry of selenium-containing biological molecules. References 99 and 96 report measurements of some biologically important molecules, including dimethyl selenide, dimethyl diselenide; and the selenoamino acids, selenocysteine, selenocystine, selenocysteamine, and selenomethionine. The ^{77}Se T_1 values and chemical shifts determined in those studies are given in Tables 3 and 4 respectively.

A pH ~5, 50% aqueous D_2O solution of selenocysteine (96) has a ^{77}Se resonance with a linewidth at half height of 150 Hz. The large linewidth is attributed to the effect of exchange between various protonated species. The ^{77}Se chemical shift of selenocysteamine (96) was found to be very sensitive to pH variations; the chemical shift of the selenol-deprotonated form, $^{-}SeCH_2CH_2NH_3^{+}$ is ~150 ppm to lower frequency of the protonated species, $HSeCH_2CH_2NH_3^{+}$. Chemical exchange between these species is reflected in linewidths at resonance half height of up to 13 Hz. From chemical shift values measured at four pH values, the pK_A for the selenohydryl proton was estimated to be 5.0. The ^{77}Se spectrum of D-,L-selenocystine (96) shows 2 resonances of equal intensity, due to the

D-,D-(L-,L-) and D-,L-(L-,D-) isomers. The resonances are triplets which result from the two bond coupling with the methylene protons. For selenomethionine (99), a single resonance with a half height linewidth of ~30 Hz was observed, due to unresolved multiplet patterns. The normal excretory product of selenium, the trimethylselenonium ion, has a ^{77}Se resonance at 140 ppm in $CDCl_3$ and 298 ppm in D_2O indicating the sensitivity of the ^{77}Se chemical shift to environmental differences in the two solvents (96).

Due to the sensitivity of the selenium nucleus to its environment and the relatively large chemical shift range illustrated by the results presented in Table 4, ^{77}Se NMR would appear to be an excellent probe with which to study the reactions in which selenium is involved.

Resonances have not been observed for ^{77}Se naturally present in proteins, for example, the ^{77}Se in glutathione peroxidase; however, the feasibility of observing the ^{77}Se spectra of selenium-containing derivatives of proteins has been demonstrated. For example, ^{77}Se containing derivatives can be formed by reacting thiol-containing proteins with the selenium analog of Ellman's reagent, [6,6'-diselenobis(3-nitrobenzoic acid), DSNB] (IV) (102). Large biomolecules such as denaturated and reduced ribonuclease-A and denaturated and reduced lysozyme were

reacted with DSNB to form the selenenyl sulphide derivatives.

(IV)

The ^{77}Se NMR spectra for the resulting selenenyl sulphides were presented. ^{77}Se NMR spectra of these compounds were obtained at 38.2 MHz from preparations which were enriched to the level of 90 to 94% in the ^{77}Se isotope. Selected ^{77}Se chemical shifts from this study can be found in Table 4. Low molecular weight biomolecules containing thiol groups such as cysteine, acetylcysteine, and glutathione were also reacted with DSNB and their ^{77}Se chemical shifts reported.

In a very recent study (83), ^{77}Se NMR spectroscopy was used to investigate the binding of (phenylselenyl)-acetate to the enzyme α-chymotrypsin. This is the first reported application of ^{77}Se NMR spectroscopy to study the binding of a protein-selenoligand complex. The 38.17 MHz

^{77}Se NMR spectra show a 39.8 ppm chemical shift difference between the free ligand and the α-chymotrypsin-(phenylselenyl)acetate complex. Linewidths of the resonances varied with different enzyme-ligand ratios.

In this thesis, ^{77}Se NMR has been used to directly monitor and characterize selected selenium-containing compounds; the results are reported in Chapters 4 and 5. The results include ^{77}Se NMR shifts and spin lattice relaxation times for selected molecules. Also, the sensitivity of the ^{77}Se chemical shift to the chemical state of the molecule is demonstrated in a study of the acid-base behaviour of diselenodiethanoic acid. The ^{77}Se chemical shift of this resonance was measured at various pH values and the chemical shift-pH data were used to evaluate the pK_A values of carboxylic acid groups of the diselenide.

2. Methylmercury(II)-Selenol Binding

The function of glutathione peroxidase is to catalyze the conversion of a variety of hydroperoxides, including organic hydroperoxides and hydrogen peroxide, that are formed *in vivo* to alcohols, thus protecting sensitive tissues from peroxidative damage. Previous studies (74) have shown that glutathione peroxidase is inhibited by a variety of metal ions, including cadmium(II), zinc(II), silver(I), mercury(II), cobalt(II), and lead(II). It has

been proposed that inhibition is due to binding of the metal ions to the selenium at the active center of the enzyme.

In methylmercury poisoning, the target organ is the brain. It is well established that methylmercury(II) [$CH_3Hg(II)$] causes lysis of cells of the central nervous system, however, the cause of cell lysis has not been established at the molecular level. If catalysis of the conversion of organic hydroperoxides to alcohols were inhibited, for example, due to binding of $CH_3Hg(II)$ to glutathione peroxidase, the cell membrane would be more susceptible to peroxidative damage by reaction with hydroperoxides. However, for $CH_3Hg(II)$ to bind to the selenium of glutathione peroxidase, the selenolate donor group (RSe^-) would need to exhibit a high affinity for $CH_3Hg(II)$ relative to the affinity of $CH_3Hg(II)$ for the thiolate group (RS^-), since sulphydryl groups are present in cells at higher concentrations than is glutathione peroxidase. For example, in erythrocytes, the concentration of glutathione, hemoglobin and glutathione peroxidase are ~2 mM, 5 mM and 10^{-6} M, respectively. Although indirect observation of the complexation of $CH_3Hg(II)$ to small selenohydryl ligands has been reported (154), the complexation of $CH_3Hg(II)$ with the selenium of glutathione peroxidase or with small selenium-containing

molecules has not been quantitatively characterized and thus it is not possible to predict if $CH_3Hg(II)$ will bind to glutathione peroxidase *in vivo*.

In Chapter 3 of this thesis, the results of a study of the binding of $CH_3Hg(II)$ by a variety of selenols are presented. These compounds were selected as models for selenium-containing molecules which may occur naturally, including the selenium of the reduced form of glutathione peroxidase. The structure and names of the selenols studied are shown in Figure 2. First, the acid-base chemistry of the selenols is characterized in Chapter 3 and then their $CH_3Hg(II)$ complexes are characterized in terms of their formation constants.

3. The Reaction of Thiols with Diselenides and the Reaction of Selenols with Disulphides

Little information is available at the molecular level about the mechanisms by which selenium becomes incorporated into biological systems. The reaction of thiols with diselenide and the reaction of selenols with disulphides do not appear to have received much attention as possible metabolic pathways for the incorporation of selenium into proteins. Although these reactions (equations 4 and 5) are known to occur (89-90),

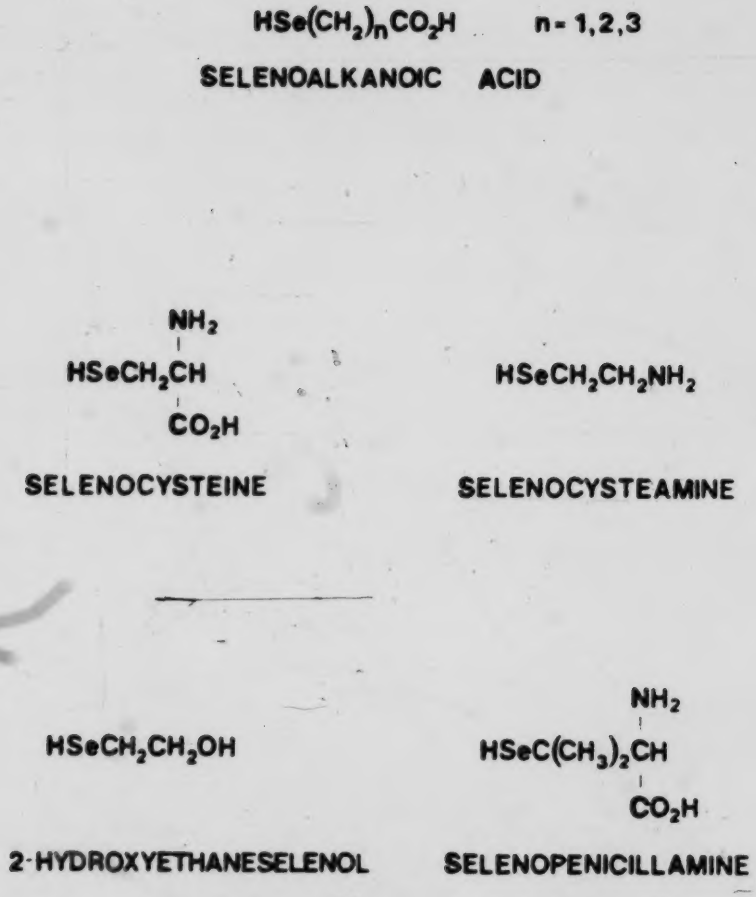


Figure 2. Structures and names of selenohydryl ligands.

$$RSH + R'SeSeR' \rightleftharpoons RSSeR' + R'SeH \quad [4]$$

$$RSH + RSSeR' \rightleftharpoons RSSR + R'SeH \quad [5]$$

neither the mechanism of the reactions nor the nature of the product, selenenyl sulphide, RSSeR' which is formed has been investigated in detail. Only one report of the reaction of selenol with disulphide is known (91). In this report, selenenyl sulphides are described as being relatively unstable, in that they undergo reaction with excess thiol and/or selenol to form the more stable diselenide and disulphide. Compounds which contain aromatic R and/or R' groups are reported to be more stable than those for which R and/or R' are alkyl groups.

Since this appears to be a plausible pathway by which organoselenium compounds in the form of selenoamino acids can be incorporated into a biological system, it was decided to examine these reactions in more detail and to characterize the solution chemistry of the selenenyl sulphides which are formed. ^{1}H and ^{77}Se NMR spectroscopy were use as the measurement techniques, as this technique can provide molecular level information regarding these reactions. That a selenenyl sulphide form of oxidized glutathione peroxidase may be present further merits the characterization of these compounds. Results from ^{1}H and

^{77}Se NMR measurements on diselenides and selenenyl sulphides are presented in Chapter 4.

4. Reaction of Selenite with Thiols

Inorganic selenium in the form of selenite is widely used as a dietary selenium supplement in cases of selenium deficiency (84) and in studies on the effects of selenium in the biological system (87-88). In 1941, Painter (9) studied the conversion of inorganic selenium into organo-selenium compounds. Selenium dioxide, SeO_2, was reacted with thiols according to equation 6. The products of the reaction are a disulphide, RSSR, and a bis(alkylthio)-

$$4RSH + SeO_2 \rightarrow RSSeSR + RSSR + 2H_2O \qquad [6]$$

selenide, RSSeSR. From equation 6, the overall stoichiometric ratio of the reactants is 4 moles of thiol to 1 mole of selenium. Later, instead of using SeO_2, Ganther (10) studied the reaction of selenious acid (H_2SeO_3) with thiols. In these studies, the bis(alkylthio)selenides were established as genuine chemical species, and the 4:1 thiol-selenium ratio was shown to indeed be the overall stoichiometry.

The thiols whose reaction with selenious acid has been studied include 2-mercaptoethanol, cysteine, glutathione,

ribonuclease A, and co-enzyme A. Although this reaction is suggested in Figure 1 to be a metabolic pathway for selenite in mammals, very little information has been reported about the mechanism of the reaction and the nature of the selenium-containing product, RSSeSR. Bis(alkylthio)selenides have been found to be unstable at alkaline pH's, decomposing to the disulphide and elemental selenium. In a more recent study, the bis(alkylthio)selenides formed by the reaction of selenite with mercaptoethanol, dodecane thiol, N-acetyl cysteine, D-penicillamine, and thionein (85), were examined using X-ray photoelectron spectroscopy. The unusual stability of bis(D-penicillamine)selenide against decomposition up to pH 7 was noted and attributed to the steric hindrance of the methyl groups. A value of "near +1" was assigned to the formal oxidation state of selenium in bis(alkylthio)selenides. The solution chemistry of bis(alkylthio)selenides, including their acid-base chemistry, have not been reported.

The most recently reported studies of the reaction of selenite with thiols are those of Kice et al. (14), who used conventional and stopped-flow spectrophotometry to measure rates of reaction. The mechanism of the reaction of selenite with thiols was studied using simple alkane thiols such as n-butyl thiol and t-butyl thiol as

models. The reaction was found to occur in two stages when an excess of thiol was used. A more detailed coverage of the mechanism of reaction is given in Chapter 5 of this dissertation. Although the details of the reaction of the n-butyl thiol and t-butyl thiol appear to have been characterized, those for the reaction of thiol groups in biological molecules with selenite have not. The results of ^{1}H and ^{77}Se NMR studies of the reaction of thiols with selenite are presented in this thesis.

^{1}H NMR spectra were obtained for solutions containing bis(alkylthio)selenides and the acid-base chemistry of selected bis(alkylthio)selenides was characterized. The ^{77}Se NMR spectra for selected symmetrical, RSSeSR and asymmetrical, RSSeSR' molecules formed by the reaction of the appropriate thiols with selenite are presented, followed by results obtained from ^{77}Se NMR studies of the acid-base chemistry of selected bis(alkylthio)selenides.

CHAPTER 2

EXPERIMENTAL

A. Chemicals

1. Selenium Compounds

The diselenides D-,L-selenocysteine and selenocystamine dihydrochloride were obtained from Sigma Chemical Co. and were used as received. They contained no impurities which could be detected in 360 MHz ^1H NMR spectra of 10 mM D_2O solutions of the diselenides. Diselenodiethanoic acid was prepared by acid hydrolysis of the selenocyanate (HO_2CCH_2SeCN), which was prepared by the reaction of 2-bromoethanoic acid with potassium selenocyanate in acetone (118). Diselenodiethanoic acid was purified by recrystallization from benzene-ethyl acetate. 3-,3'-diselenodipropanoic acid was prepared from 3-bromopropanoic acid and aqueous sodium diselenide (119) by Dr. A.P. Arnold and the late S.-Y. Ha, and purified by recrystallization from hot water. 4-,4'-Diselenodibutanoic acid (120) and bis(2-hydroxyethane)diselenide (121) were prepared by hydrolysis of the selenosulphates, which were prepared by reaction of the corresponding bromides and potassium selenosulphate in water. 4-,4'-

Diselenodibutanoic acid was purified by reprecipitation several times from a bicarbonate solution by adding dilute sulphuric acid. Bis(2-hydroxyethane)diselenide is an orange oil, and was purified by fractional distillation under reduced pressure. Selenopenicillamine diselenide was synthesized by preparing Se-benzylselenopenicillamine from isopropylidene-2-methyl-5(4)-oxazolone and benzylselenol in pyridine according to the method of Draguet and Renson (122). The benzyl group was removed from the Se-benzylselenopenicillamine by reaction with sodium in liquid ammonia to give selenopenicillamine, from which selenopenicillamine diselenide was obtained by overnight air oxidation in the presence of a catalytic amount of iron(III) chloride at pH 9. Contaminating dibenzyldiselenide was removed by extraction with benzene, and then the aqueous phase was acidifed to pH 6 to precipitate the selenopenicillamine diselenide. The ^{1}H NMR spectrum of the pale yellow product showed it to be contaminated with valine. Two reprecipitations from alkaline solution were required to obtain selenopenicillamine diselenide free of impurities which could be detected by ^{1}H NMR.

2. Sulphur Compounds

Mercaptoacetic acid and 3-mercaptopropanoic acid

(Aldrich Chemical Co.) were purified by fractional distillation under reduced nitrogen pressure. 4-mercaptobutanoic acid was synthesized from γ-butyrolactone and thiourea (123) and was purified by fractional distillation under reduced nitrogen pressure (b.p. 89-90, 0.05 mm Hg). D-,L-cysteine (Sigma Chemical Co.) was used as received. Cysteamine hydrochloride (Aldrich Chemical Co.) was sublimed *in vacuo* to purify it from the disulphide, cystamine, which the ^1H NMR spectrum of a 10 mM solution indicated was present as an impurity. Resonances for cystamine could not be detected in the ^1H NMR spectrum of a 10 mM solution of sublimed material. 2-Mercaptopropanoic acid, D-penicillamine, D-,L-penicillamine and glutathione (Sigma Chemical Co.) were used as received; their respective ^1H NMR spectra showed no evidence of disulphide impurities. D_2O solutions of thiols were prepared immediately prior to their use.

3. Other Chemicals

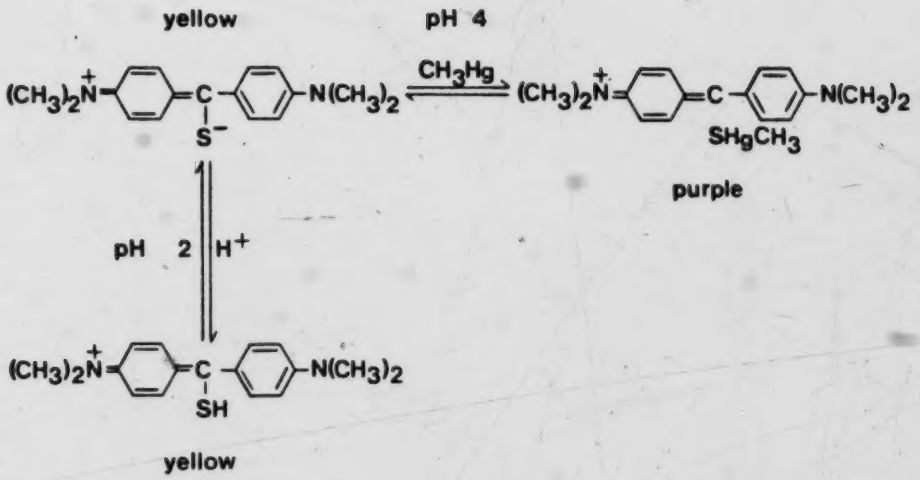
Sodium thiosulphate was used as received. Michler's thioketone (bis[4-(dimethylamino)phenyl]methanethione) was obtained from Eastman. The potassium nitrate used for ionic strength control was recrystallized from water. Potassium deuteroxide (40%) and deuterated nitric acid (68%) (Sigma Chemical Co.) were diluted with deuterium

oxide (MSD Isotopes Merck Frosst) to give 1-2 M solutions. Tertiary butanol (Fisher Scientific), formic acid, sodium formate, acetic acid, sodium acetate, imidazole, disodium hydrogen phosphate, monopotassium dihydrogen phosphate and sodium selenite (Sigma Chemical Co.) were research grade and were used as received.

B. Preparation and Standardization of Methylmercury(II) Solution

Methylmercury(II) iodide (Alfa Division, Ventron Corp.) was converted to a stock solution of CH_3HgOD by reaction with Ag_2O (124). The CH_3HgOD concentration of the stock solution was determined by using the titrimetric method described by Erni (125,126). In this method, methanol is added to the CH_3HgOD solution to give a 50% aqueous methanol solution, which is then titrated with thiosulphate at pH ~ 4. The thiosulphate was standardized by titration with iodate as described in reference 128. The end point in the $CH_3Hg(II)$-thiosulphate titration was located using Michler's thioketone (MTK) (bis[4-(dimethylamino)phenyl]methanethione) as indicator.

The indicator reacts with $CH_3Hg(II)$ as represented by equation 7 to form a 1:1 $CH_3Hg(II)$-MTK complex which has a formation constant of log K_f = 8.27 (127). Titration with



[7]

thiosulphate displaces the MTK as described by equation 8. A sharp colour change from purple to yellow is obtained at the equivalence point.

$$CH_3Hg(MTK)^+ + S_2O_3^{2-} \rightleftarrows CH_3Hg(S_2O_3)^- + MTK \qquad [8]$$

In the procedure used, a 10 mL aliquot of the stock CH_3HgOD solution was diluted 10 times with water. 10 mL aliquots of this diluted solution were then diluted with 10 mL of 1 M pH 4.0 acetate buffer and 20 mL methanol, and titrated with 0.020 M $Na_2S_2O_3$ using MTK indicator (4 drops

0.1% MTK in acetone). Titrations were done in triplicates. Two stock solutions were prepared, one having a concentration of 0.1478 M with a relative standard deviation of 0.0030 and a second having a concentration of 0.7509 M with a relative standard deviation of 0.0037. The concentration of the first stock solution was re-determined 9 weeks after the first standardization to determine its stability; the concentration was found to be 0.1486 ± 0.0026 M, indicating no significant change in concentration.

Due to the toxicity of $CH_3Hg(II)$, stringent precautions were taken to avoid physical contact with the CH_3HgOD solutions by wearing PVC gloves; avoiding inhalation and working in a well-ventilated fumehood.

C. Preparation of Selenols from Diselenides

The selenohydryl ligands used in the study of $CH_3Hg(II)$ complexation were obtained by electrolytic reduction of 10 mM solutions of the corresponding diselenides in D_2O containing 0.3 M KNO_3. Magnetically stirred 10 mM diselenide solutions were deaerated with nitrogen (or argon) and then reduced at a mercury pool electrode (area ~12.5 cm^2) which was held at a potential of -1.0 V with respect to the saturated calomel electrode.

The construction of the electrolysis cell is shown in Figure 3. The cell is sealed with a Teflon cap which has drilled holes to hold the various components mentioned below. The auxiliary electrode consists of a double salt bridge (0.3 M KNO_3 in D_2O) with inverted ground glass sleeve junctions separating an Ag/AgCl electrode in saturated KCl from the diselenide solution. A saturated calomel electrode was used as the reference electrode. An inert gas inlet capillary and an outlet hole allow the reduction cell to be maintained under an inert atmosphere during the reduction. A pH microcombination electrode was used for pH measurement. A sample can be withdrawn from the cell via a hole which is stoppered during the reduction process.

A Princeton Applied Research Model 174A polarographic analyzer was used as the potential source. The half wave potentials for the reduction of the diselenides were determined polarographically to be in the range -0.5 V to -0.7 V over the pH range 3 to 10, agreeing with those reported by Nygard (129-131). Reductions were carried out at -1.0 V vs the saturated calomel reference electrode. From current-time plots, of the type shown in Figure 4, the half-times for the constant potential reduction of 25 mL of 10 mM diselenide solution, were determined to be 30-40 minutes.

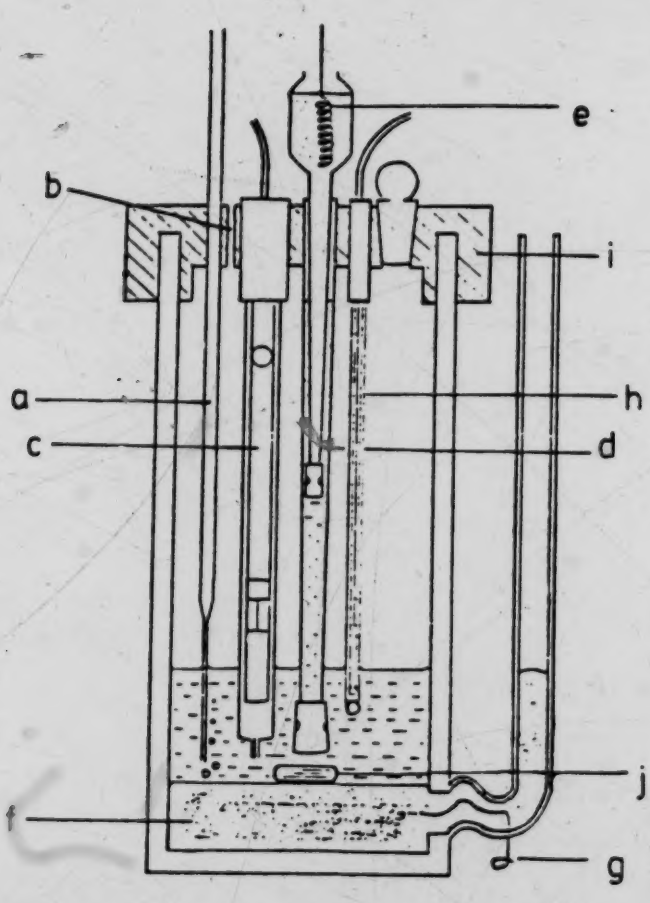


Figure 3. A schematic diagram of the reduction cell used to convert diselenides to selenols. The cell has a capacity of 20-50 mL. The specific components are:

(a) inert gas inlet capillary,
(b) gas outlet hole,
(c) saturated calomel reference electrode,
(d) double salt bridge of 0.3 M KNO_3/D_2O with ground glass sleeve junctions separating
(e) the auxiliary electrode (Ag/AgCl, saturated KCl) from the solution (k),
(f) mercury pool working electrode with
(g) platinum wire contact lead,
(h) pH microcombination electrode,
(i) tight fitting Teflon cap containing stoppered hole for sample withdrawal. The solution is continuously stirred by the magnetic bar (j).

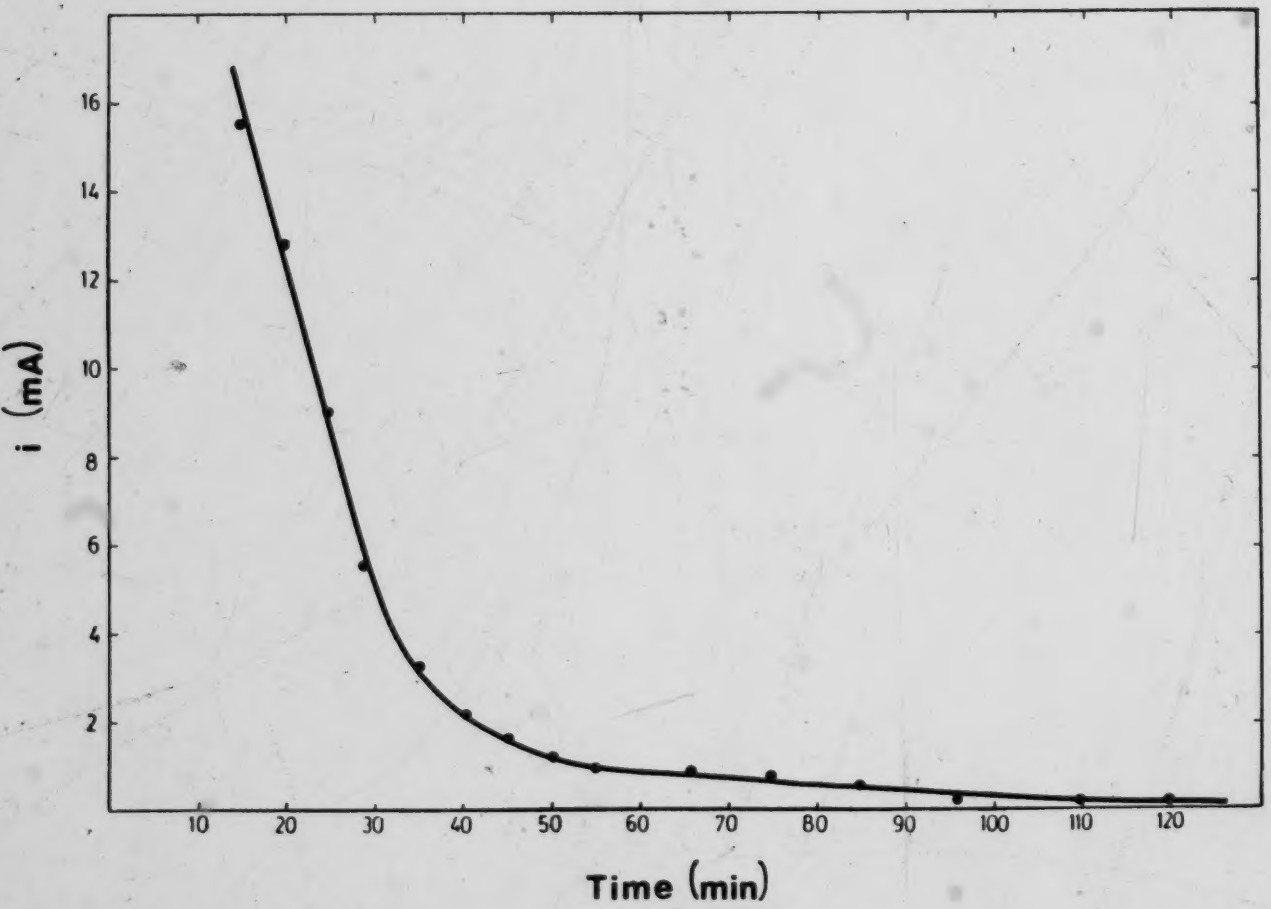


Figure 4. Current-time curve for the reduction of a D_2O solution of 5.03 mM diselenodipropanoic acid in 0.3 M KNO_3 to selenopropanoic acid at -1.0 V versus saturated calomel electrode.

The pD of the diselenide solutions was adjusted to ~8-9 and reduction carried out until a colourless solution was obtained, at which time the pD had risen to ~11. The complete reduction of the diselenide was confirmed by obtaining a ^1H NMR spectrum of the reduced solution in which the resonances for the selenol were not detectably broadened due to exchange between the selenol and diselenide forms (equation 9).

$$R^*Se^- + RSeSeR \rightleftarrows RSe^- + R^*SeSeR \qquad [9]$$

At pH values where the selenol is deprotonated, the presence of 1% diselenide causes visible broadening of the selenolate signals. The presence of residual diselenide in the solution could also be detected in ^1H NMR spectra of solutions at a sufficiently low pD that the selenolate is completely protonated. Under these conditions, exchange is slow on the NMR time scale, and separate resonances can be observed for the selenol and diselenide forms. When reduction was complete, aliquots of the selenolate solution were withdrawn into an inert gas-purged titration cell for use in pK determinations and for use in the $CH_3Hg(II)$-selenohydryl complexation studies.

D. Instrumentation

1. pH Measurements

All pH measurements were made with either an Orion Model 701A or a Fisher Accumet pH meter, equipped with a standard glass electrode (Philips GAT 130) and an inverted glass sleeve, double junction saturated calomel reference electrode (Philips R44/2-SD/1). The usual reference electrode which has KCl filling solution and a frit for a liquid junction was not used because formation of CH_3HgCl in the frit clogs the liquid junction. To avoid formation of CH_3HgCl, a 0.3 M potassium nitrate solution was used as the outer electrolyte of the double junction reference electrode. A two-point calibration of the pH meters was routinely performed with 0.05 molal phthalate (pH 4.008) and 0.005 molal phosphate (pH 6.865) buffer solutions which were freshly made according to NBS specifications (132).

pH measurements for D_2O solutions were converted to pD values (142) with the relation:

$$pD = \text{pH meter reading} + 0.4 \qquad [10]$$

In this thesis, those pH meter readings which were obtained in D_2O solutions but were not converted to pD

values will be indicated by the notation pH*.

2. ^{1}H NMR Measurements

^{1}H NMR spectra were measured at 360-MHz on a Bruker WM 360 NMR spectrometer operating in the pulse/Fourier transform mode. Measurements were made at the ambient probe temperature of 25 ± 2°C. The ^{1}H chemical shifts were measured relative to the methyl resonance of t-butanol (TBA), which was added as an internal chemical shift reference, and are reported relative to the methyl resonance of sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS); the methyl resonance of TBA is 1.2365 ppm to high frequency from that of DSS.

Free induction decays for a spectral width of 4000 Hz were collected in 16K of data points. Quadrature detection was employed. Control of spectrometer operation and processing of data were done with an Aspect 2000 computer system using Bruker software.

All ^{1}H NMR measurements were made using 5-mm o.d. NMR tubes.

3. ^{77}Se NMR Measurements

^{77}Se NMR spectra were obtained at 68.68 MHz on a Bruker WM-360 NMR spectrometer operating in the pulse/Fourier transform mode. Spectra were measured at an ambient probe temperature of 25 ± 2°C when no ^{1}H

decoupling was used. When spectra were measured with ^{1}H decoupling, the sample tube was cooled with air which had passed through a cooling coil which was placed in a dewar of dry ice. The probe temperature was maintained at 25°C with a variable temperature unit.

Because of the very wide chemical shift range (~3000 ppm) of ^{77}Se (92-94,96,103,109), it is not possible to use a single compound as a chemical shift reference for the entire ^{77}Se chemical shift range. Thus, chemical shift measurements were made by setting the spectrometer reference frequency at a fixed value corresponding to the chemical shift of the ^{77}Se resonance from a solution of 8.4 M H_2SeO_3. The chemical shift of the resonance for an 8.4 M H_2SeO_3 solution is 1282 ppm relative to the resonance for $(CH_3)_2Se$, which has been assigned a chemical shift of 0 ppm (109). A spectrum of the 8.4 M H_2SeO_3 solution was obtained prior to each series of ^{77}Se measurements to ensure a proper lineshape and good resolution of resonances in subsequent spectra, as well as to set the spectrometer reference frequency.

For some measurements, for instance, the measurement of spectra by the inversion recovery pulse sequence (180°-τ_1-90°-acquisition) for the determination of spin-lattice relaxation times, the pulse lengths for 90° and 180° flip angles were required. The ^{77}Se 90° pulse length was

measured using the chemical shift reference sample of H_2SeO_3. A typical series of spectra obtained in a pulse width determination are shown in Figure 5. The value of 24 μs was selected as the pulse length for a 90° pulse and 48 μs for a 180° pulse.

Solutions used in the measurement of ^{77}Se NMR spectra had concentrations of 20 mM-0.2 M. In most cases, solutions were made up in 90% v/v aqueous D_2O containing 0.3 M KNO_3. For almost all cases, extensive decomposition was observed at higher concentrations for selenium-containing compounds prepared by the reaction of thiols with selenite; thus 0.2 M was used as the upper concentration limit.

^{77}Se NMR spectra were obtained by Fourier transforming free induction decays which have been multiplied by an exponential function for sensitivity enhancement; a line broadening of 3 to 5 Hz was usually used.

4. Potentiometric Titrations

Potentiometric titrations were carried out with an automated computer-controlled titrator (133). The titrator has the following components:

1. IBM PC which controls titrant delivery and the measurement of pH, and calculates equilibrium

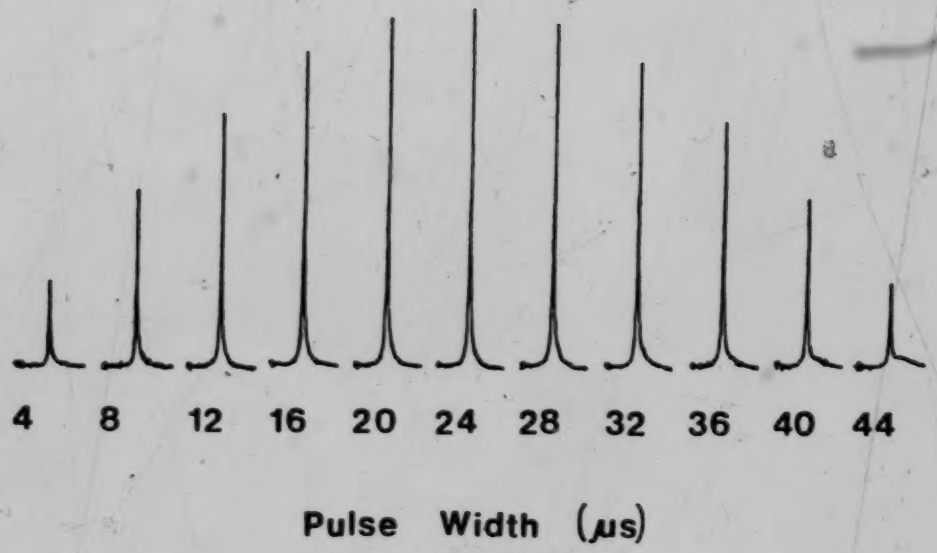


Figure 5. Determination of the pulse length for a 90° flip angle for ^{77}Se NMR at a frequency of 68.69 MHz. The ^{77}Se resonances are from 8.4 M H_2SeO_3 in D_2O. The resonance was measured as a function of pulse width as indicated in the figure. From these data the pulse width for a 90° flip angle was determined to be 24 μs.

constants from titration data.

2. Mettler DV11 autoburette with a motorized piston for titrant delivery.
3. Orion 701A pH meter.
4. A double-walled titration cell which is sealed with a Teflon stopper holding Philips GAT 130 low resistance glass and Philips R44/2-SD/1 inverted glass sleeve, double junction saturated calomel reference electrodes, an inert gas inlet tube and outlet hole, a Pederson 5 μL micropipette serving as titrant delivery tube and a capped hole from which samples may be withdrawn if required.

The solution was magnetically stirred and continuously purged with inert gas throughout the length of the titration.

The procedure involved adding the solution being titrated to the titration cell. The titration program was then initiated on the IBM PC, where automatic operation takes place which involves pH measurement and recording, followed by the addition of titrant; the process is repeated until completion of the titration according to the conditions specified in a parameter file. A typical titration went from pD 11 to pD 2, and a total titrant volume of 0.6 mL was added in 0.01-0.02 mL increments.

The entire titration took from 20-40 minutes for completion, and 40-80 titration data points were obtained. The titration data were then processed using the FORTRAN program MINIQUAD (134-136). Reference-133 gives details for the implementation and use of this potentiometric titration set-up.

E. Determination of Acid Dissociation Constants

Titrant solutions of ~2 M potassium deuteroxide (KOD) and deuterated nitric acid (DNO_3) were prepared in D_2O. The KOD titrant was standardized by potentiometric titration of primary standard grade potassium hydrogen phthalate (Fisher Scientific). The DNO_3 titrant was then standardized by titration of the standardized KOD solution. A 1 mL aliquot of KOD was diluted in 50 mL water and titrated with DNO_3. The standardization of these titrants was carried out potentiometrically using the IBM PC titrator set-up described in Section D (4).

Two methods were employed for the determination of acid dissociation constants:

1. Potentiometric titration, which was used for all thiol and selenol pK^D_A determinations.
2. Variation of the chemical shift of ^1H or ^{77}Se resonances with pD. This method was used for

$CH_3Hg(II)$-selenohydryl complexes, $CH_3Hg(II)$-sulphydryl complexes, mercaptoacetic acid, selected diselenides, alkyl selenenyl sulphides, and bis(alkylthio)-selenides.

As pH measurements were obtained with a glass and reference electrodes in D_2O solutions, the pD value was calculated using equation 10. The activity of the deuterium ion (D_3O^+) is then defined as

$$a_D = 10^{-pD} \qquad [10a]$$

The equations used below for the determination of pK^D_A values by the computer program, MINIQUAD and the non-linear least squares curve fitting routine, KINET, are derived with the quantity a_D.

1. Potentiometric Titration

Solutions used for the potentiometric titration of selenohydryl ligands contained an initial concentration of 5 mM diselenide in 0.3 M KNO_3 in D_2O; the diselenide was converted to the selenol form by electrolysis in the reduction cell as described above. A 20 mL aliquot of the selenolate solution was transferred from the electrolysis cell in an argon-flushed 20 mL pipette into the inert gas-

purged titration cell. As the initial pD was >11, dilute standardized DNO_3 was used as titrant. Occasionally, the acidic selenolate solution at the end of this first titration was titrated to high pD again with standardized KOD. As the titrants were ~2 M, only small amounts of titrant were added from the computer-controlled Mettler DV11 motorized burette; typically, ~0.6 mL of titrant was used for the complete titration.

Solutions for the determination of the pK^D_A values of the thiol ligands contained 5 mM thiol in 0.3 M KNO_3 in D_2O. The potentiometric titrations were carried out on 20 mL aliquots and titrant was added to cover the pD range 2-12.

The computer-program MINIQUAD (134-136), which was written for the determination of metal-ligand stability constants from potentiometric titration data, was used for the evaluation of acid dissociation constants from potentiometric titration data. This program evaluates the overall formation constants, β_{mlh}, for the complex species, $M_mL_lH_h$, which forms according to the overall formation equilibrium

$$mM + lL + hH_3O^+ \rightleftarrows M_mL_lH_h + hH_2O \qquad [11]$$

β_{mlh} is defined as

$$\beta_{mlh} = \frac{[M_m L_l H_h]}{[M]^m [L]^l [a_H]^h} \qquad [12]$$

With a simple modification, the potentiometric titration data for a ligand (e.g. a selenol) can be evaluated by MINIQUAD, to yield acid dissociation constants. For a D_2O solution containing ligand only, [M] = 0 and m = 0, and the above equations can be rewritten as

$$L + dD_3O^+ \rightleftarrows LD_d + dD_2O \qquad [11a]$$

and

$$\beta_{01d} = \frac{[LD_d]}{[L][a_D]^d} \qquad [12a]$$

The relationship between the overall formation constant and the acid dissociation constant for a monoprotic acid is

$$\beta_{011} = \frac{[LD]}{[L][a_D]} = \frac{1}{K^D_A} \qquad [13]$$

i.e. $\log \beta_{011} = pK^D_A$.

For a diprotic acid,

$$\beta_{011} = \frac{[LD]}{[L][a_D]} = \frac{1}{K^D_{A,2}} \qquad [14]$$

i.e. $\log \beta_{011} = pK^D_{A,2}$ and

$$\beta_{012} = \frac{[LD_2]}{[L][a_D]^2} \qquad [15]$$

Thus,

$$\beta_{012} \cdot K^D_{A,2} = \frac{[LD_2]}{[LD][a_D]} = \frac{1}{K^D_{A,1}} \qquad [16]$$

giving

$$\begin{aligned} \log \beta_{012} &= pK^D_{A,1} + pK^D_{A,2} \\ &= pK^D_{A,1} + \log \beta_{011} \end{aligned} \qquad [17]$$

β_{011} and β_{012} are correlated and therefore the standard deviations are calculated by MINIQUAD using the relationship for correlated variables a,b:

$$\sigma^2_{a\pm b} = \sigma^2_a + \sigma^2_b - 2\rho_{ab}\sigma_a\sigma_b \qquad [18]$$

where ρ_{ab} is the correlation coefficient obtained from the non-linear least square procedure.

2. Chemical Shift Titration

In the NMR titration experiments to determine acid dissociation constants for $CH_3Hg(II)$ complexes or for thiols, a solution containing 10 mM 1:1 $CH_3Hg(II)$ complex or thiol in 0.3 M KNO_3 in D_2O was titrated with acid or base and samples removed at appropriate pD values for NMR measurements. Five μL of t-butanol was added for an internal chemical shift reference. The 1:1 $CH_3Hg(II)$ complex solution was prepared as described in Section F of this chapter.

The determination of acid dissociation constants by NMR involves obtaining chemical shift data as a function of pH for resonances from nuclei which are near the acidic group. Typically, the procedure involved placing a 20 mL solution in an inert gas-flushed polarographic cell which was fitted with a plastic top holding the glass and reference electrodes, an inert gas inlet tube, a titrant delivery tube and capped holes from which samples could be withdrawn or replaced into the titration cell.. The pD of the solution was adjusted with small quantities of either 2 M DNO_3 or 2 M KOD and a pH meter reading taken after ~60 s when equilibrium had been established, i.e. when the pH meter readings were observed not to vary by more than ±0.002 pH units min^{-1}. The NMR spectrum was then obtained, during which time the pD* of the remaining

solution in the cell was adjusted and the next NMR sample withdrawn. The first sample was returned to the titration cell, with the aid of a plastic syringe fitted with a length of plastic tubing, and the procedure repeated. NMR spectra were measured at a total of 24-40 pD values in order that the chemical shift changes over the relevant pD range were well defined. The pH meter calibration was rechecked at the end of each titration.

Proton exchange is generally fast on the NMR time scale, and thus exchange averaged resonances are observed. The chemical shifts of the exchange averaged resonances are the weighted average of the chemical shifts of the two forms between which the exchange occurs. Acid dissociation constants can be obtained from chemical shift versus pD data by fitting the data to the appropriate equilibrium model; in this work, the data were fitted using the non-linear least squares curve fitting program KINET. The equations relevant for the extraction of acid dissociation constants depends upon whether the acid is monoprotic, diprotic, or polyprotic.

a. Monoprotic Acids

The acid dissociation scheme for a monoprotic acid in D_2O can be written as

$$DA + D_2O \rightleftharpoons D_3O^+ + A^- \qquad [19]$$

and the acid dissociation constant defined as

$$K^D_A = \frac{[a_D][A]}{[DA]} \qquad [20]$$

Exchange between the DA and A^- forms is fast on the NMR time scale, and thus resonances for nuclei in close proximity to the deprotonation site are exchange averaged and located at a chemical shift which is intermediate between those of DA and A^-. The actual chemical shift at any pD depends on the population of each form as given by the relation

$$\delta_{obsd} = \delta_{DA}\alpha_{DA} + \delta_A\alpha_A \qquad [21]$$

where δ_{obsd} is the observed chemical shift, δ_{DA} and δ_A are the chemical shifts of the protonated and deprotonated species, respectively. α_{DA} is the fraction of the total acid which is present as DA:

$$\alpha_{DA} = \frac{[DA]}{[DA] + [A]} \qquad [22]$$

α_A is the fraction present as the deprotonated species and

is given by

$$\alpha_A = \frac{[A]}{[DA] + [A]} \qquad [23]$$

Re-expression of the above α's in terms of K^D_A gives

$$\alpha_{DA} = \frac{a_D}{a_D + K^D_A} \qquad [22a]$$

and

$$\alpha_A = \frac{K^D_A}{a_D + K^D_A} \qquad [23a]$$

where a_D is the activity of deuterium ion as defined in equation 10a. Substitution of the above equations into the equation for the observed chemical shift yields:

$$\delta_{obsd} = \frac{a_D\,\delta_{DA} + K^D_A\,\delta_A}{a_D + K^d_A} \qquad [24]$$

K_A was determined from chemical shift versus pD data by fitting the data to equation 24. When the limiting chemical shifts, δ_{DA} and δ_A, could be obtained directly from the chemical shift versus pD data, K_A is the only unknown parameter and can be easily extracted from the chemical shift-pD data by fitting the data to the above

equation. When the limiting chemical shifts could not be obtained directly, e.g. because of overlapping resonances, δ_{DA} and/or δ_A were also treated as unknown parameters and were evaluated together with K_A by fitting the data with the non-linear least square computer program KINET.

b. Diprotic Acids

The overall acid dissociation scheme in D_2O solution is represented by

$$D_2A + D_2O \underset{}{\overset{K^D_{A,1}}{\rightleftharpoons}} DA + D_3O^+ \qquad [25]$$

$$DA + D_2O \underset{}{\overset{K^D_{A,2}}{\rightleftharpoons}} A + D_3O^+ \qquad [26]$$

and the acid dissociation constants are defined as

$$K^D_{A,1} = \frac{[DA][a_D]}{[D_2A]} \qquad [27]$$

$$K^D_{A,2} = \frac{[A][a_D]}{[DA]} \qquad [28]$$

Resonances for the diprotic acids studied in this work are all affected by the state of the protonation of both acidic groups. The chemical shifts observed for such resonances is given by

$$\delta_{obsd} = \delta_{D_2A}\alpha_{D_2A} + \delta_{DA}\alpha_{DA} + \delta_A\alpha_A \qquad [29]$$

where α_{D_2A}, α_{DA}, and α_A are the fractional concentrations of the various species and δ_{D_2A}, δ_{DA} and δ_A are the chemical shifts of these species, respectively. The fractional concentration can be expressed in terms of K^D_A's and a_D, leading to the expression:

$$\delta_{obsd} = \frac{a_D^2 \cdot \delta_{D_2A} + a_D \cdot K^D_{A,1} \cdot \delta_{DA} + K^D_{A,1} \cdot K^D_{A,2} \cdot \delta_A}{a_D^2 + a_D \cdot K^D_{A,1} + K^D_{A,1} \cdot K^D_{A,2}} \qquad [30]$$

In most cases encountered in this research, the two limiting chemical shifts, δ_{D_2A} and δ_A, can be obtained directly from chemical shift versus pD data.

All acid dissociation constants determined in this work for ligands in D_2O solutions have the notation pK^D_A while those determined for D_2O solutions of $CH_3Hg(II)$-selenol complexes have the notation pK'^D_A.

F. Preparation of Solutions of 1:1 Methylmercury(II)-Selenol Complexes

A 20 mL aliquot of ~ 10 mM selenol solution was

withdrawn from the reduction cell with an argon-flushed pipette and transferred into an argon-purged polarographic cell. The solution was stirred continuously with a magnetic stirring bar and purged with argon. Five μL of t-butanol was added, followed by an amount of standardized CH_3HgOD corresponding to ~95% of the selenol content. The solution pD was 12 ± 0.1. At this pD, exchange of selenol between its free and complexed forms is fast on the NMR time scale.

Two NMR methods were used to determine the attainment of a 1:1 $CH_3Hg(II)$:selenol ratio:

1. Variation of the chemical shifts of the exchange averaged selenol resonances with the addition of standardized CH_3HgOD titrant.
2. Variation of the chemical shifts of resonances for an added indicator molecule such as thiouracil.

The complexity of the selenol resonances determined which of the two methods was used; a first order spectrum for the selenol renders the first method applicable, while the second method was used when the selenol spectrum was complex due to second order spin-spin coupling effects.

1. Method I

A 0.5 mL sample of the $CH_3Hg(II)$-selenol solution was

withdrawn into an argon-flushed 5-mm o.d. NMR tube, the NMR spectrum obtained, and the sample returned to the cell. A 10 μL volume of CH_3HgOD was added to the solution, a sample withdrawn and its NMR spectrum obtained. This procedure was repeated until there was no change in the chemical shift of the selenol resonances being monitored. A plot of chemical shift versus the amount of standardized CH_3HgOD added shows an increase in chemical shift up to the point when a 1:1 metal-ligand ratio was reached, and then levels off beyond this point. Figure 6 shows data for a typical titration.

The titration curve shows two distinct regions, and straight lines can be drawn through the points in each of these portions. The 1:1 equivalence point is taken as the point of intersection of these lines. The excess CH_3HgOD added beyond the equivalence point was calculated from the volume added and then an equivalent amount of selenol was added to give a 1:1 solution.

2. Method II

The indicator molecule method requires the addition of small quantities of another molecule which can complex with $CH_3Hg(II)$, but with a smaller formation constant so that this indicator reacts with $CH_3Hg(II)$ only after titration of selenol with $CH_3Hg(II)$ is complete. This

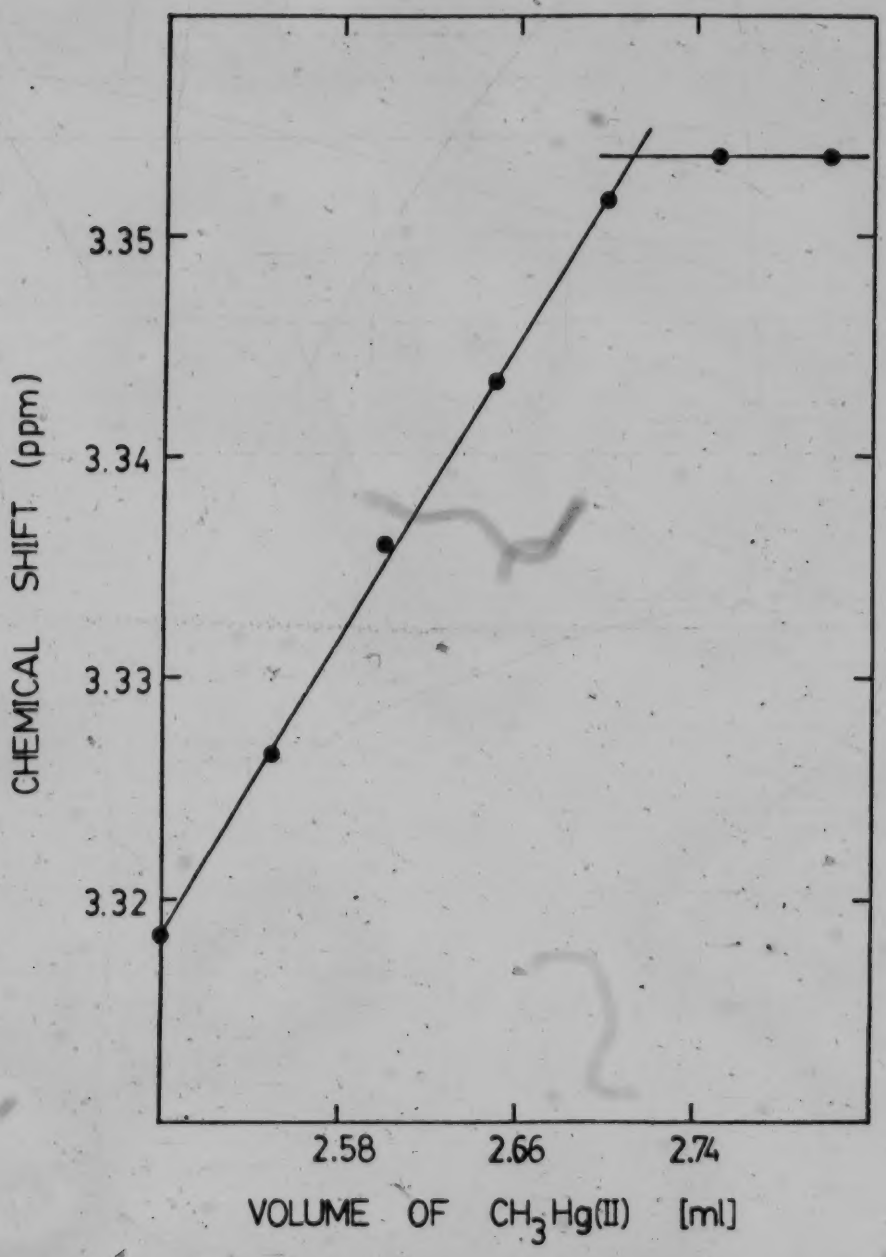
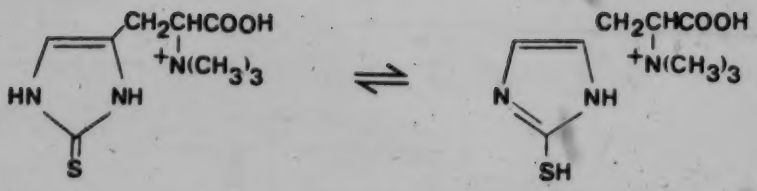


Figure 6. Chemical shift of the methylene resonance of selenoethanoic acid during its titration with standard CH_3HgOD (0.148 M) to prepare a 1:1 $CH_3Hg(II)$:selenoethanoic acid solution. From these data the concentration of selenoethanoic acid was determined to be 20.04_2 mM.

method has been used previously to prepare 1:1 $CH_3Hg(II)$-thiol solutions (137,138). In that procedure, ergothioneine was used as the indicator. The log K_f for ergothioneine is 13.7 ± 0.2 (137), whereas the log K_f for $CH_3Hg(II)$-thiol complexes is 15.0 - 16.0. Ergothioneine could not be used in this work because its methyl resonance would overlap the resonances to be used to determine $CH_3Hg(II)$-selenol formation constants.

Three other possible indicator molecules were examined: methimazole(2-mercapto-1-methylimidazole), 2-thiohydantoin and 2-thiouracil. These molecules were chosen because they, in common with ergothioneine, undergo thiol-thione tautomerism at the sulphur atom. The sulphur atom is still the $CH_3Hg(II)$ binding site, but with a smaller formation constant than for binding to a thiolate or a selenolate group, because of the thiol-thione tautomerism.

Methimazole was found to be unsuitable because its methyl resonance interferes in the chemical shift region of interest, while thiohydantoin was found to be unsuitable due to its decomposition at pD values above 8.5. Thiouracil was found to be stable throughout the pD range of interest and its resonances occur to higher frequency than the region of interest in the subsequent formation constant experiments.



ergothioneine

methimazole

2-thiohydantoin

2-thiouracil

The accuracy of the results obtained using thiouracil as an indicator molecule was determined by using it to locate the 1:1 equivalence point in the titration of mercaptoacetic acid with $CH_3Hg(II)$. Thiouracil was added at a concentration of 3 mM in the test experiment. After each addition of $CH_3Hg(II)$, the chemical shift of both the exchange-averaged methylene resonance of MAA and the highest frequency resonance of thiouracil were determined. The 1:1 equivalence points were determined from plots of chemical shift versus the amount of CH_3HgOD added for both resonances. Results are shown in Figure 7. The equivalence points obtained from the sets of data agree to 2%, confirming the suitability of thiouracil as an indicator molecule in this work.

Whenever an indicator molecule was required for the determination of the 1:1 equivalence point, it was added to the initial approximately equimolar $CH_3Hg(II)$-selenol

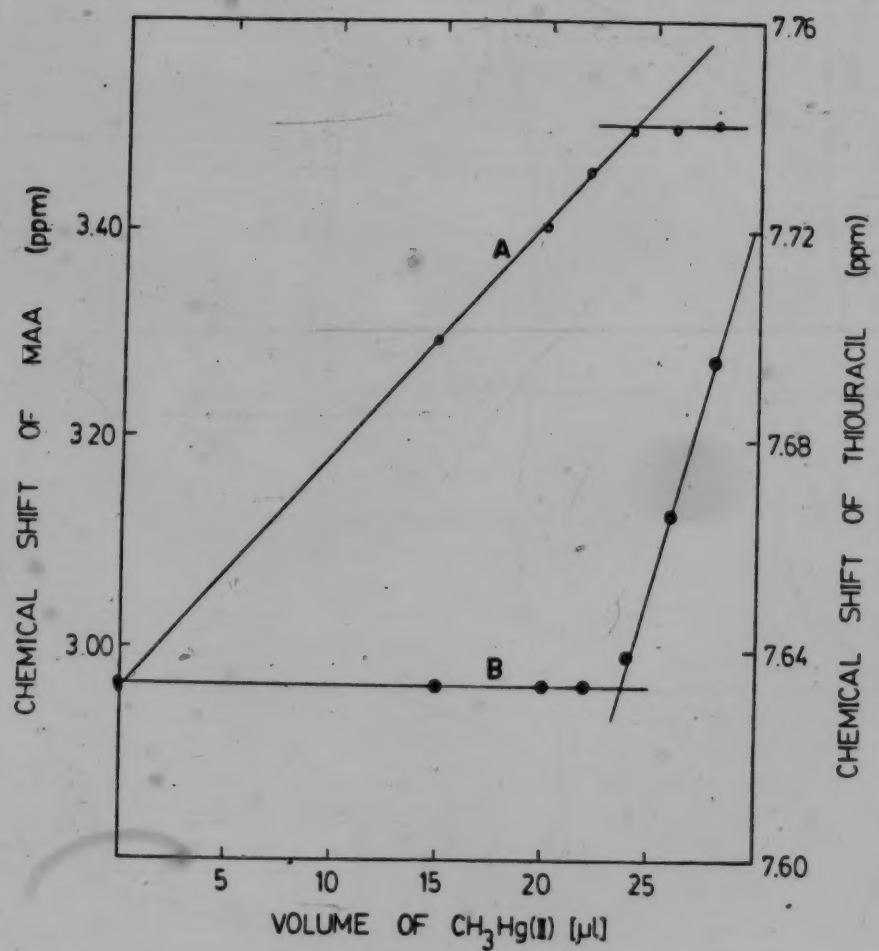


Figure 7. Chemical shift of [A]: the methylene resonance of MAA and [B]: the highest frequency resonance of thiouracil during the titration of a solution containing MAA and thiouracil with standard CH_3HgOD (0.751 M). From the end point in A, the concentration of MAA is calculated to be 8.91_7 mM, while it is calculated to be 8.93_6 mM from the end point obtained from curve B.

solution, at a final concentration of 3 mM.

G. Preparation of $CH_3Hg(II)$-Selenol-Thiol Solutions

Solutions containing approximately equimolar $CH_3Hg(II)$, selenol and mercaptoacetic acid were required for the competition equilibria studies. Approximately equimolar solutions were required in order to avoid preferential complexation of $CH_3Hg(II)$ by one ligand or the other. If this occurs, the exchange averaged chemical shift of the ligand resonances, which is the quantity measured to determine the formation constant, will lie close to that of either free or bound ligand and it would be difficult to determine the equilibrium constant accurately from chemical shift data. $CH_3Hg(II)$-selenol solutions prepared at pD ~12 as described in the previous section were used. A fresh solution of 20 mM mercaptoacetic acid (MAA) in 0.3 M KNO_3/D_2O was prepared and deaerated for 10 minutes for each competition reaction studied. The appropriate volume of this thiol solution was taken into an argon-flushed pipette and added to the 1:1 $CH_3Hg(II)$-selenol reaction solution with constant stirring and purging with argon. The final solution mixture had approximately equimolar $CH_3Hg(II)$, selenol and thiol.

^1H NMR spectra were measured for the 1:1:1 solution as a function of pD. A 0.5 mL sample of this solution (pD ~12) was withdrawn into a 5 mm o.d. NMR tube and its ^1H NMR spectrum obtained. Meanwhile, the pD of the remaining solution was adjusted and another 0.5 mL sample withdrawn for NMR analysis. The first sample was returned to the reaction cell and the pD adjusted for the next sample. Typically, a total of 20-30 samples were taken to cover the pD range 2-12. Data for displacement constant determination were obtained by following the chemical shift of the exchange-averaged methylene resonance of MAA as the pD of the solution was varied.

H. Preparation of Solutions and Procedure Used to Study the Reaction of Thiols with Selenite

Solutions of 10-20 mM thiol and 2.5-50 mM selenite were prepared in D_2O containing 0.3 M KNO_3 immediately prior to their use. A stock buffer solution containing 0.3 M KNO_3, 50 mM buffer and 5-10 μL t-butanol for ionic strength, pH control, and chemical shift reference, respectively, was made in D_2O, and deaerated before use.

In a typical experiment, 0.5 mL of a 10 mM sodium selenite solution was pipetted into a 2 mL volumetric flask. 1.0 mL of a 20 mM thiol solution was added with a

graduated pipette and the solution made up to the mark with the stock buffer solution. The solution was quickly mixed and a 0.5 mL sample removed and transferred into a 5-mm o.d. NMR tube which had been flushed with inert gas. Spectra of the reaction mixture were obtained at relevant time intervals after reaction was initiated. For each thiol, the reaction was carried out over the pD range 1.0-8.0. The thiol and disulphide resonances were assigned by comparison with spectra of authentic samples which were measured at the same pD as used in the reaction of thiol with selenite.

I. Computer Programs

The non-linear least squares program KINET (139) was used for the evaluation of acid dissociation constants and for the determination of displacement constants in the $CH_3Hg(II)$-selenol-mercaptoacetic acid experiments. The program fits experimental data to a proposed model. The variables in the experimental data used in this thesis are pD and chemical shift.

The computer program KINET was developed for curve fitting and equation solving problems. In this work, the version used (KINFIT) is for fitting a data set to a user specified function. This function is input as an equation

in a subroutine and initial estimates are given for the unknown parameters. The program then predicts the chemical shift at the experimental pD values. The measured chemical shift is the dependent variable and is subtracted from the calculated chemical shift to give a residual. The curve fitting is effected by iteratively minimizing the sum of squares of the residuals by adjustments of the unknown parameters. Initial estimates for the parameters must be user supplied and should be reasonably good to ensure convergence in the iterative minimization routine. In this work, the initial estimates could generally be obtained by inspection of the experimental chemical shift versus pD data set. The final output gives the set of parameter values which best fits the data set. These are reported with overall standard deviations and a final sum of squares of the residual. The magnitudes of these provide indications of the goodness of the fit.

Potentiometric titration data were fitted with a computer program MINIQUAD (134-136), as described earlier. Species distribution calculations were done by using a modified version (141) of a published computer program, COMICS (COncentration in Mixtures of Metal Ions and Complexing Species) by Perrin and Sayce (140).

CHAPTER 3

THE COMPLEXATION OF METHYLMERCURY(II) BY SELENO-AMINO ACIDS AND RELATED MOLECULES

A. Introduction

The biological chemistry of methylmercury(II) [$CH_3Hg(II)$] has received widespread interest since the realization of its potential public health hazard. Extensive studies on the complexation of $CH_3Hg(II)$ have been carried out, and the results are summarized in several review articles (143,144). From the point of view of the behaviour of $CH_3Hg(II)$ in biological systems, complexation of $CH_3Hg(II)$ by biological sulphydryl compounds is of particular interest. It is well established that $CH_3Hg(II)$-thiol complexes have large formation constants (127,145,146). Also, ^1H NMR studies of the binding of $CH_3Hg(II)$ in intact human erythrocytes have provided direct experimental evidence for the complexation of $CH_3Hg(II)$ in an intact biological system (147).

The complexation of $CH_3Hg(II)$ by selenium is also of interest since selenium is present, as a selenocysteinyl

group, at the active site of the reduced form of the enzyme glutathione peroxidase (GSH-Px) (150). The ability of this enzyme to protect cells from peroxidative damage (151) would presumably be affected if a significant fraction of the enzyme is $CH_3Hg(II)$ complexed, under toxic situations. An intriguing aspect of selenium and $CH_3Hg(II)$ toxicity is that various selenium containing compounds have significant protective effects in animal studies of $CH_3Hg(II)$ poisoning (148,149). The molecular basis for this effect has yet to be established.

As selenium is softer in a Lewis base sense than sulphur, it could be expected to form more stable complexes with Class B metal ions such as $CH_3Hg(II)$ (152) than does sulphur. Evidence supporting this has been reported. For example, the formation constant of the $CH_3HgSeCN$ complex is larger than that of the CH_3HgSCN complex (153). A relation between the coupling constant for spin-spin coupling between the methyl protons and the mercury of $CH_3Hg(II)$, $^2J_{^{199}Hg-^1H}$ and the formation constant of $CH_3Hg(II)$ complexes has also been observed: the magnitude of the coupling constant decreases as the formation constant of the $CH_3Hg(II)$ complexes increases. This observation has been used to provide qualitative information regarding the relative magnitudes of the formation constants of $CH_3Hg(II)$ complexes (154-155).

A communication has been recently published for the complexation of $CH_3Hg(II)$ with D-,L-selenomethionine (77), allowing comparison with $CH_3Hg(II)$-methionine formation constants determined by Fairhurst and Rabenstein (78). At pH above 8.0, the $CH_3Hg(II)$ binding site is the amino group on both amino acids, while at pH's less than 1.0, the thioether/selenoether is the binding site. Methionine has a log K_f value of 1.94 at pH 0.5 (78), while selenomethionine has a log K_f value of 3.73 at pH 0.8 (77). This indicates that the selenoether group forms a more stable complex with $CH_3Hg(II)$ than the analogous thioether donor. No formation constants for $CH_3Hg(II)$-selenohydryl complexes have been reported for a quantitative comparison with those of $CH_3Hg(II)$-sulphydryl complexes.

The purpose of the research described in this chapter is to characterize the binding of $CH_3Hg(II)$ to selenium-containing amino acids and related molecules. This was accomplished by measuring formation constants for the $CH_3Hg(II)$-selenolate complexes. Because the formation constants are large, they were determined by using a competitive reaction procedure (equation 31), in which the position of the displacement equilibrium is measured. Specifically, a thiolate ligand was used as the competitive ligand. As the formation constant for CH_3HgSR

and CH_3HgSeR complexes are expected to be similar, the equilibrium position is

$$CH_3HgSR + RSe^- \underset{}{\overset{K_d}{\rightleftharpoons}} CH_3HgSeR + RS^- \qquad [31]$$

expected to be such that it can be determined accurately from ^1H NMR chemical shift measurements. The displacement constant, K_d is defined as

$$K_d = \frac{K_{f,CH_3HgSeR}}{K_{f,CH_3HgSR}} \qquad [32]$$

In most cases studied, the selenol ligand has other acidic groups, so that the CH_3Hg-selenol complex may exist in various protonated forms. For example, in the case of selenocysteine, Figure 8 shows the acid-base and $CH_3Hg(II)$ complexation equilibria. In this work, formation constants are reported for the reaction of $CH_3Hg(II)$ with the fully deprotonated ligands. However, this formation constant together with acid dissociation constants for the acidic group on the ligand suffice to describe the complex chemistry of the system at any pH.

The competition reaction approach has been used previously in this laboratory to measure large formation constants. In this method, it is necessary to select a

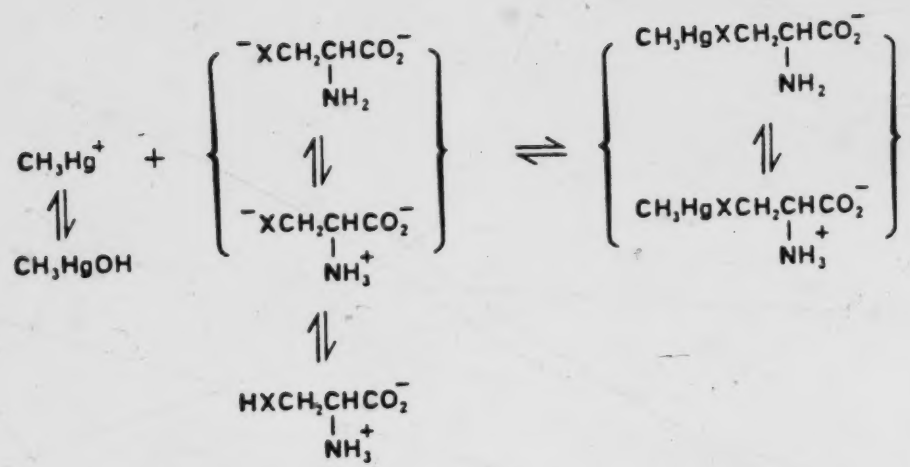


Figure 8. The competing complexation and protonation reactions occurring in aqueous solutions of $CH_3Hg(II)$ and selenocysteine (X = Se) and $CH_3Hg(II)$ and cysteine (X = S).

competing ligand which will react with $CH_3Hg(II)$ with a formation constant comparable in magnitude to the one to be determined, so that the displacement equilibrium constant is of a magnitude which can be determined accurately from chemical shift data, for example, displacement equilibrium constants in the range 0.1-10.

B. Selection of Mercaptoacetic Acid as the Competing Ligand

Mercaptoacetic acid (MAA) was chosen as the competing ligand because

1. It has relatively simple acid-base behaviour in both the free and $CH_3Hg(II)$ complexed forms.
2. Its ^1H NMR spectrum consists of only a single resonance line whose chemical shift is dependent upon the protonation state of the carboxylic acid and the thiol groups, and upon the degree of complexation at the thiol group.

The acid-base chemistry of mercaptoacetic acid in D_2O is described by the following equations:

$$DSCH_2COOD + D_2O \underset{}{\overset{K^D_{A,1}}{\rightleftharpoons}} DSCH_2COO^- + D_3O^+ \qquad [33]$$

$$DSCH_2COO^- + D_2O \underset{}{\overset{K^D_{A,2}}{\rightleftharpoons}} {}^-SCH_2COO^- + D_3O^+ \qquad [34]$$

The mixed concentration-activity acid dissociation constants are defined as:

$$K^D_{A,1} = \frac{[DSCH_2COO^-] \cdot a_D}{[DSCH_2COOD]} \qquad [35]$$

$$K^D_{A,2} = \frac{[{}^-SCH_2COO^-] \cdot a_D}{[DSCH_2COO^-]} \qquad [36]$$

Curve A in Figure 9 shows the variation of ^1H chemical shift of the methylene protons as a function of pD. The fully protonated species, $DSCH_2COOD$ is present at and below pD 2.0. The carboxylic acid group begins to deprotonate as pD is raised. The chemical shift observed is intermediate between that of the fully protonated $DSCH_2COOD$ and the carboxylic acid deprotonated species, $DSCH_2COO^-$. This chemical shift is the weighted average value dependent upon the population of the two species. Above pD 9.0, deprotonation of the sulphydryl group occurs and the observed chemical shift is intermediate between that of $DSCH_2COO^-$ and ${}^-SCH_2COO^-$. Under conditions of fast

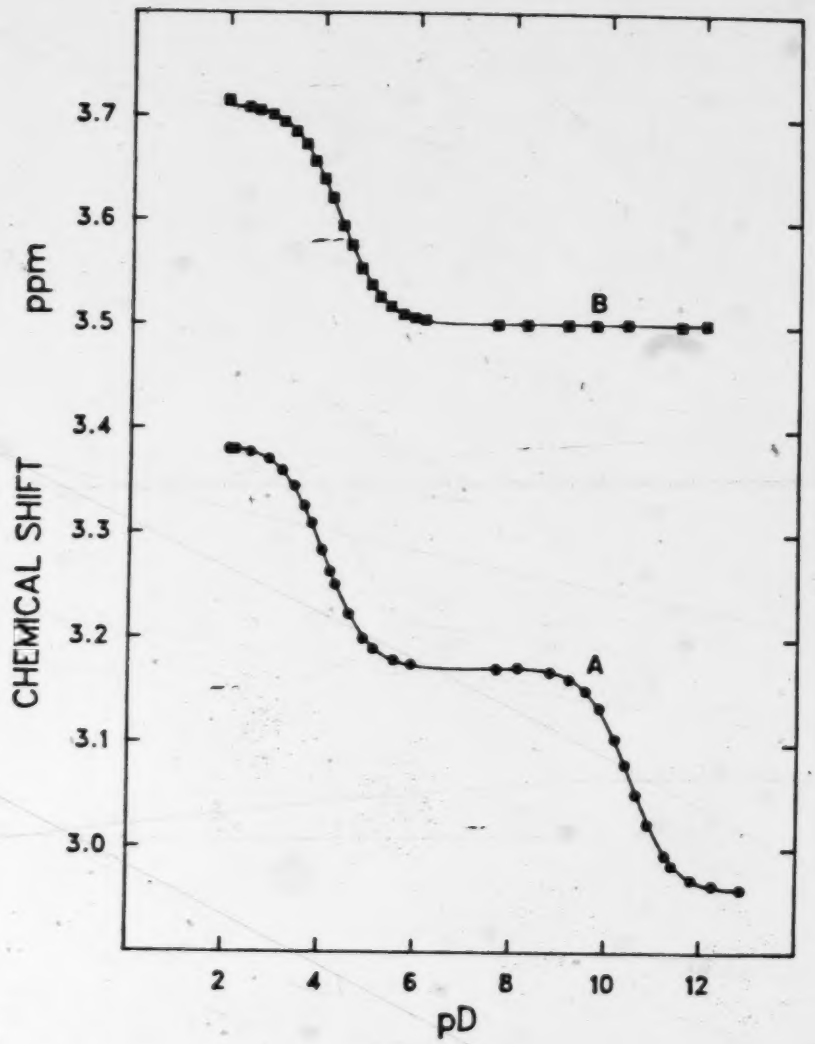


Figure 9. Chemical shift-pD data for A: free mercaptoacetic acid and B: $CH_3Hg(II)$-complexed mercaptoacetic acid.

exchange, as is the case for the experimental conditions used in this work, the observed chemical shift of the free ligand at any pD is the weighted average value, given by:

$$\delta_{obsd} = \delta_1\alpha_1 + \delta_2\alpha_2 + \delta_3\alpha_3 \qquad [37]$$

where δ_{obsd} is the observed chemical shift and δ_1, δ_2, δ_3 are the chemical shifts for the $DSCH_2COOD$, $DSCH_2COO^-$, and $^-SCH_2COO^-$ forms of mercaptoacetic acid, respectively. α_1, α_2, and α_3 are the fractions of the ligand in the three forms. Values were determined for $pK^D_{A,1}$ and $pK^D_{A,2}$ by fitting chemical shift versus pD data to this model as described in Chapter 2. The values obtained and the chemical shifts δ_1, δ_2, and δ_3 are given in Table 5.

For the $CH_3Hg(II)$ complex, the acid-base chemistry in D_2O is described by equation 38:

$$CH_3HgSCH_2COOD + D_2O \xrightleftharpoons{K'^D_A} CH_3HgSCH_2COO^- + D_3O^+ \qquad [38]$$

and the acid dissociation constant is given by:

$$K'^D_A = \frac{[CH_3HgSCH_2COO^-] \cdot a_D}{[CH_3HgSCH_2COOD]} \qquad [39]$$

Table 5. Chemical shifts and acid dissociation constants of mercaptoacetic acid.

Species	Chemical Shift (ppm)	Reference
$DSCH_2COOD$	3.3827 ± 0.0002	This work
	3.390	146
$DSCH_2COO^-$	3.1724 ± 0.0002	This work
	3.184	146
$^-SCH_2COO^-$	2.9607 ± 0.0003	This work
	2.968	146

$pK^D_{A,1}$	$pK^D_{A,2}$	Type[a]	Reference
3.68_4 ± 0.002	10.11_6 ± 0.003	M	This work[b]
3.50 ± 0.01	10.08 ± 0.02	M	146[c]
3.40	10.07	T	156[d]
3.27	10.05	T	157[d]
3.53	10.40	T	158[d]

[a] T = thermodynamic, M = mixed.

[b] 25°C and ionic strength 0.3 M in D_2O.

[c] Under the same conditions as in this work but in H_2O.

[d] Corrected to ionic strength 0.3 M in H_2O.

Curve B in Figure 9 shows the variation of the ^1H chemical shift of the methylene protons of the 1:1 $CH_3Hg(II)$-mercaptoacetate complex with pD. This chemical shift is the weighted average of the protonated, CH_3SCH_2COOD, and deprotonated, $CH_3HgSCH_2COO^-$, forms of the complex and at any pD is given by:

$$\delta'_{obsd} = \delta'_1\alpha'_1 + \delta'_2\alpha'_2 \qquad [40]$$

where δ'_{obsd} is the observed chemical shift in a solution of 1:1 $CH_3Hg(II)$-thiol complex and δ'_1 and δ'_2 are the chemical shifts for the protonated and deprotonated forms of the complex. α'_1 and α'_2 are the fractions of protonated and deprotonated forms of the complex. The pK'^D_A value of this complex was estimated from the chemical shift-pD data as described for a monoprotic acid in Chapter 2. The pK'^D_A and the chemical shifts of the CH_3Hg-mercaptoacetic acid complex are given in Table 6.

C. Determination of Acid Dissociation Constants of Selenols

In general, the acid-base dissociation constants of selenohydryl ligands have not been determined. In the few instances where they have been determined, they have been

Table 6. Chemical shifts and acid dissociation constants for the mercaptoacetic acid complex of methylmercury(II).

Species	Chemical Shift (ppm)[a]	Reference
CH_3HgSCH_2COOD	3.7089 ± 0.0009	This work
	3.715	146
$CH_3HgSCH_2COO^-$	3.5016 ± 0.0006	This work
	3.517	146

[a]For the methylene protons of complexed mercaptoacetic acid.

pK'^{D}_{A}	Type[a]	Reference
3.91 ± 0.01	M	This work[b]
3.80 ± 0.01	M	146[c]

[a]M = mixed.

[b]25°C and ionic strength 0.3 M.

[c]Under the same conditions as in this work but in H_2O.

estimated by polarography (129-130), ^{77}Se (96) and ^{1}H NMR spectroscopy, potentiometric titration (154,163) and spectrophotometric pH titration (164). The selenols studied in the above instances are selenoalkanoic acids of the homologous series $HO_2C(CH_2)_nSeH$, where n = 1-4, selenocysteine and selenocysteamine. Very few literature values for the acid dissociation constants of the other dissociable protons on the selenols used in this work are available. In the model used to determine the formation constants of the $CH_3Hg(II)$-selenol complexes, pK_A values of the selenohydryl ligands are required in order that the complexation system can be completely described.

In this work, the acid dissociation constants for all the dissociable protons on the selenols were determined potentiometrically. The acid dissociation constants were calculated by fitting potentiometric titration data to the appropriate protonation equilibria with the computer program MINIQUAD, as described in Chapter 2.

1. 2-Hydroxyethane Selenol

This selenohydryl ligand is monoprotic and therefore has the simplest acid dissociation scheme. The pK^D_A, obtained for the selenol by potentiometric titration of a solution containing 11.65 mM selenol in D_2O at 25°C and ionic strength 0.3 M is 6.60 ± 0.02. Table 7 summarizes

Table 7. Acid dissociation constants of selenohydryl compounds.

Ligand	Acid Group	pK[a,b]	Literature pK Values
Selenoethanoic Acid	CO_2D[c]	3.91 ± 0.03	
	SeD[c]	6.91 ± 0.03	7.33[d,e]
Selenopropanoic Acid	CO_2D[c]	4.70 ± 0.02	
	SeD[c]	7.32 ± 0.01	7.68[d,e]
Selenobutanoic Acid	CO_2D[c]	4.96 ± 0.02	
	SeD[c]	7.23 ± 0.02	7.78[d,e]
Selenocysteine	CO_2D	2.21 ± 0.03	2.01[f]
	SeD	5.43 ± 0.02	5.63,[d,e] 5.24[f]
	ND_3^+	10.68 ± 0.01	9.96[f]
Selenopenicillamine	CO_2D	2.24 ± 0.11	
	SeD	5.26 ± 0.07	
	ND_3^+	10.59 ± 0.04	
Selenocysteamine	SeD	5.50 ± 0.02	5.0,[g] 4.93,[d,h] 5.01[d,i]
	ND_3^+	11.45 ± 0.02	10.8[h]
2-Hydroxyethaneselenol	SeD	6.60 ± 0.02	

[a] In D_2O at 0.3 M ionic strength and 25°C.

[b] Mixed constants in terms of: "a_{D^+}" as obtained from the pH meter reading and the concentrations of the acid and its conjugate base.

[c] pK_1 and pK_2 for the $HSe(CH_2)_nCO_2H$ compounds are actually mixed constants, although the CO_2H group is somewhat more acidic than the SeH group (Kurz, J.L.; Harris, J.C. J. Org. Chem. 1970, 35, 3086).

[d] Corrected for ionic strength differences with the Davies equation (Davies, C.W. J. Chem. Soc. 1938, 2093.

[e] References 129 and 130.

[f] Reference 164.

[g] Reference 96.

[h] Reference 154.

[i] Reference 163.

the pK values determined in this work and available literature values.

2. The Homologous Selenoalkanoic Acids

The selenoalkanoic acids, $HSe(CH_2)_nCOOH$ have two acidic groups: a carboxylic acid group and a selenohydryl group. These functional groups have overlapping proton dissociation regions. Potentiometric titration data of a pD 11 solution of 4-selenobutanoic acid is shown in Figure 10. From this data, the macroscopic acid dissociation constants were determined. Although the carboxylic acid group is the more acidic compared to the selenol group, it is not so much more so that deprotonation occurs separately, as is observed in analogous mercaptocarboxylic acids.

Attempts were made to monitor the variation of the ^{77}Se chemical shift of selenoethanoic acid with pH for pK_A determination. At pH* 8.79, the ^{77}Se resonance was located at -131.51 ppm. When pH* was lowered by the addition of dilute acid, a pungent odour due to the volatile protonated selenol was detected. No ^{77}Se resonance was detected thereafter due to loss of the selenol through volatilization.

3. Selenocysteamine

Potentiometric titration data of a solution of 10.00

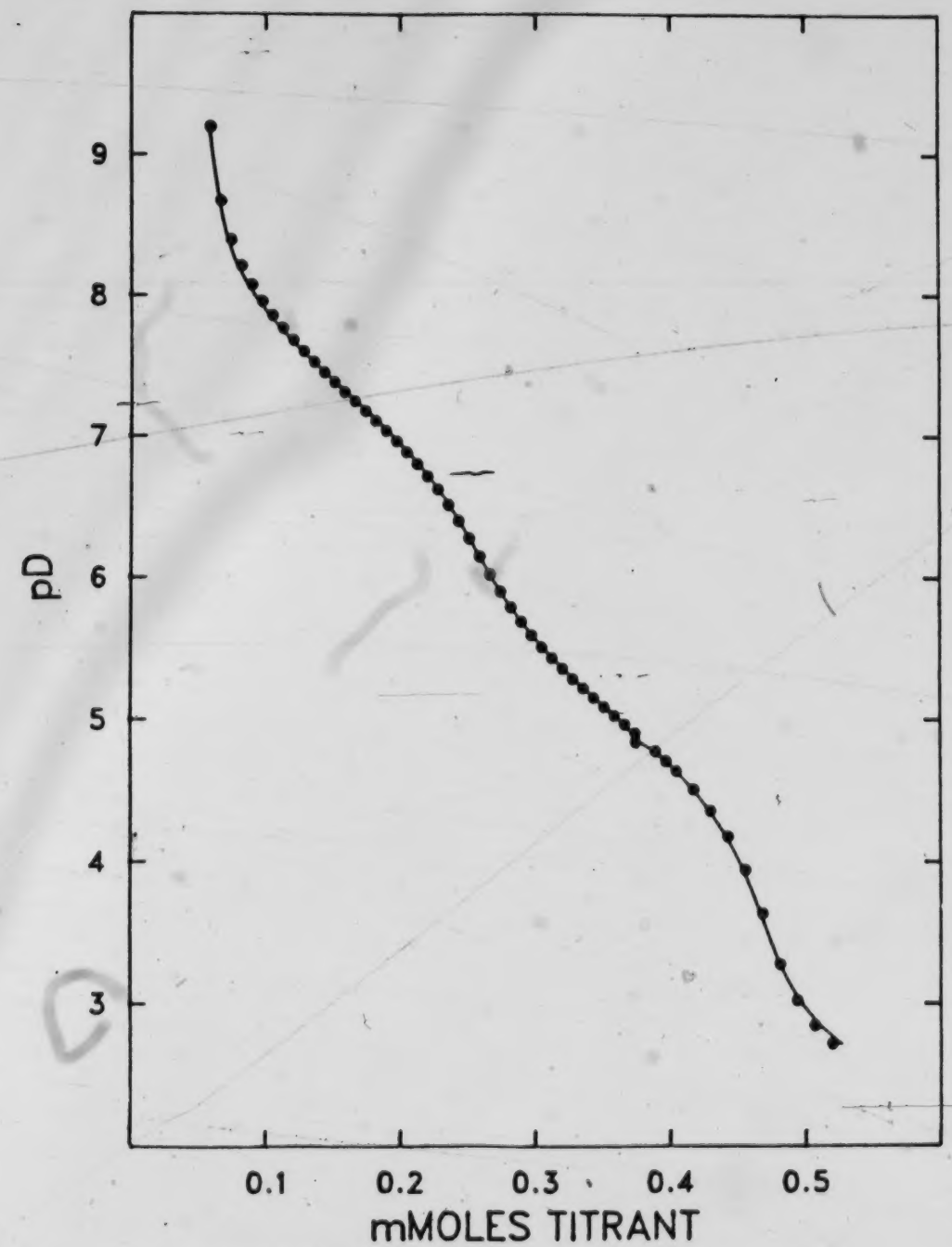


Figure 10. Potentiometric titration curve for the titration of a solution of 20.00 mM 4-selenobutanoic acid with ~1 M DNO_3.

mM selenocysteamine show that its titration occurs in two distinct regions, unlike the analogous sulphur compound, where the thiolate and amino groups are titrated over the same region. pK^{D}_{A} values were evaluated using the computer program MINIQUAD as described in Chapter 2 and are listed in Table 7.

A D_2O solution of 40.5 mM selenocysteamine was obtained by chemical reduction of selenocystamine with sodium borohydride and used to obtain ^{77}Se NMR chemical shift data for the determination of the selenohydryl pK_A. The ^{77}Se chemical shift was monitored as the pH* was varied from 3 to 8; the results are plotted in Figure 11. The fully protonated selenol, $HSeCH_2CH_2NH_3^+$, has a ^{77}Se chemical shift of -81.58 ± 0.81 ppm. As the pH* was raised the selenohydryl proton was titrated to form amino-protonated species, $^-SeCH_2CH_2NH_3^+$, which has a ^{77}Se chemical shift of -245.62 ± 0.50 ppm. The very large change in chemical shift as the selenohydryl group is titrated shows the extreme sensitivity of the ^{77}Se nucleus to its environment. The selenohydryl pK_A estimated from this set of ^{77}Se chemical shift-pH data was 5.31 ± 0.14.

4. Selenocysteine and Selenopenicillamine

Selenocysteine and selenopenicillamine are both triprotic acids. Data from the potentiometric titration

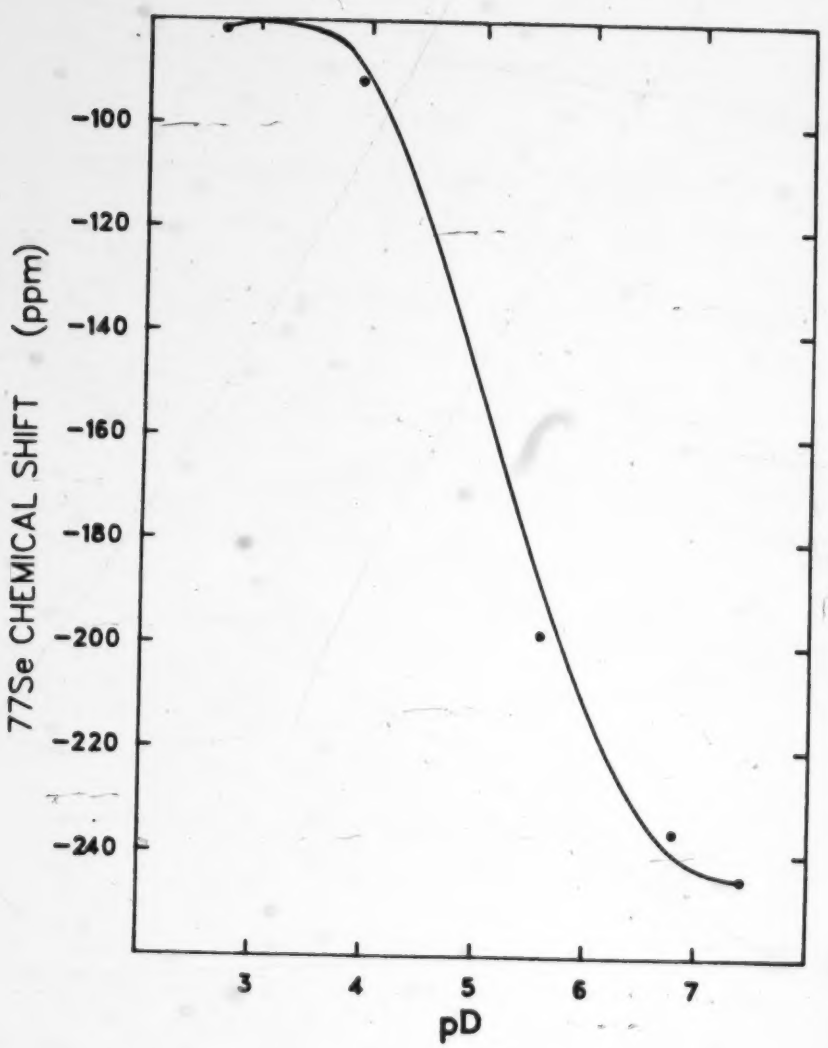
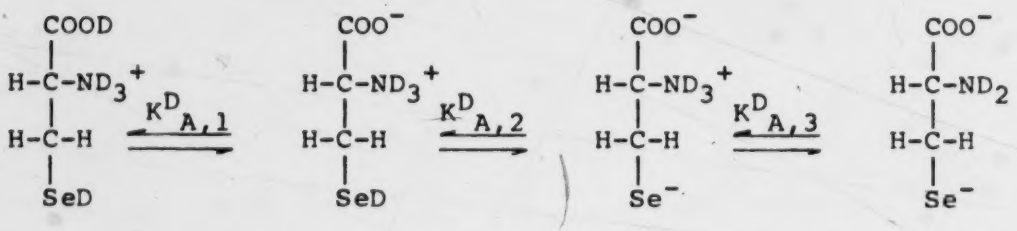


Figure 11. The chemical shift of the ^{77}Se resonance of a solution of 40.5 mM selenocysteamine as a function of pD.

of a solution of selenocysteine at pD 11 with DNO_3 is shown in Figure 12. Comparison of this titration with that for the selenoalkanoic acids indicates that protonation proceeds over three distinct regions, in the order amino, then selenolate, and finally carboxylate, as indicated by the dissociation scheme in equation 41.



[41]

The carboxylic acid group is more acidic relative to the selenol group compared to the same two groups in the selenoalkanoic acids. In contrast, the analogous sulphur compounds do not have this simplified acid dissociation scheme, as the sulphydryl and amino groups dissociate over the same region, and thus microscopic acid dissociation constants are required to fully characterize them. ^{77}Se NMR spectra of selenocysteine show a singlet resonance located at -168.5 ppm at pH* 8.86. However, when the pH was lowered and the selenohydryl group becomes protonated, the resonance becomes very broad and chemical shift-pH data could not be obtained in reasonable times to allow

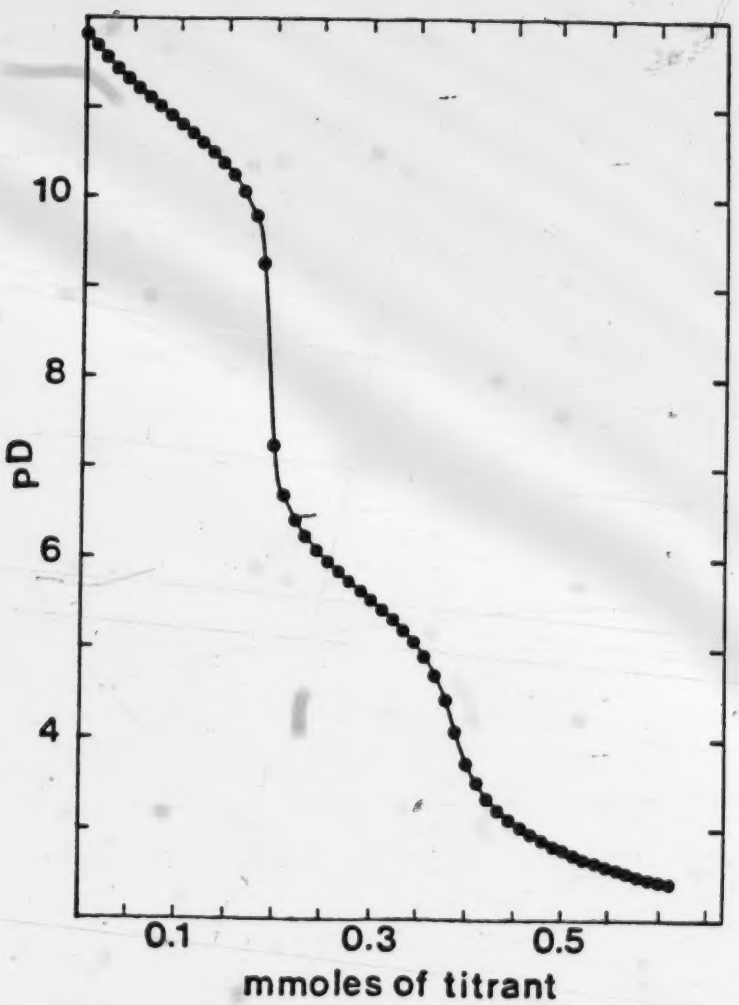


Figure 12. Potentiometric data for the titration of 20 mL of 9.81_5 selenocysteine with ~1 M DNO_3. The solid curve through the points is the theoretical titration curve calculated with pK values obtained from the data.

characterization of the acid-base chemistry by ^{77}Se NMR spectroscopy.

D. Model for the Competition Studies Between Mercaptoacetic Acid and Selenol

The model for this competition reaction is derived for $CH_3Hg(II)$ and the completely deprotonated forms of mercaptoacetic acid (MAA), $^{-}SCH_2COO^{-}$ and the selenol ligands, RSe^{-}. The displacement reaction is written as

$$RSe^{-} + CH_3HgSCH_2COO^{-} \underset{}{\overset{K_d}{\rightleftharpoons}} CH_3HgSeR + {}^{-}SCH_2COO^{-} \quad [42]$$

where

$$K_d = \frac{[{}^{-}SCH_2COO^{-}][CH_3HgSeR]}{[RSe^{-}][CH_3HgSCH_2COO^{-}]} \quad [43]$$

$$= \frac{K_{f,CH_3HgSeR}}{K_{f,CH_3Hg\text{-}MAA}} \quad [44]$$

The formation constants, K_f are defined by

$$K_{f,CH_3HgSeR} = \frac{[CH_3HgSeR]}{[CH_3Hg(II)][{}^{-}SeR]} \quad [45]$$

$$K_{f,CH_3Hg\text{-}MAA} = \frac{[CH_3HgSCH_2COO^{-}]}{[CH_3Hg(II)][{}^{-}SCH_2COO^{-}]} \quad [46]$$

The formation constant of the $CH_3Hg(II)$-mercaptoacetic acid complex has been previously determined in two separate studies (145,146); the value used in the studies described here is log $K_{f,CH_3Hg\text{-}MAA}$ = 16.92.

The displacement equilibrium constant was determined from the chemical shift of the exchange averaged resonance of mercaptoacetic acid as follows. The mass balance equations for $CH_3Hg(II)$, MAA, and the selenol are given by

$$M_T = MS_T + MSe_T \qquad [47]$$

$$S_T = S_F + MS_T \qquad [48]$$

$$Se_T = Se_F + MSe_T \qquad [49]$$

where M, S, and Se denote $CH_3Hg(II)$, mercaptoacetic acid, and the selenol, respectively; subscript F is the total free species and T, the total species present. For example, MS_T represents the sum of the concentration of the various protonated forms of the $CH_3Hg(II)$-mercaptoacetic acid complex.

A conditional displacement constant can be defined by

$$K_{dc} = \frac{[S_F][MSe_T]}{[Se_F][MS_T]} \qquad [50]$$

Experimentally, the variables are the chemical shift of the methylene protons of mercaptoacetic acid and pD. In the region of fast exchange, the chemical shift of the methylene resonance is the weighted average of the chemical shifts of the various protonated forms of free and complexed thiol, as given by:

$$\delta_{obsd} = \delta_{free} f_{free} + \delta_{comp} f_{comp} \qquad [51]$$

where $\delta_{free} = \delta_1\alpha_1 + \delta_2\alpha_2 + \delta_3\alpha_3$ as in equation 37, $\delta_{comp} = \delta'_1\alpha'_1 + \delta'_2\alpha'_2$ as in equation 40, and $1 = f_{free} + f_{comp}$. f_{free} is the fraction of free thiol which can be calculated by rearranging equation 51 to give

$$f_{free} = \frac{\delta_{obsd} - \delta_{comp}}{\delta_{free} - \delta_{comp}} \qquad [51a]$$

The total free thiol concentration can be determined from the relation

$$[S_F] = f_{free} \cdot [S_T]$$

Using the mass balance equations 47-49, the concentrations

of the various species in equation 50 can be calculated:

$$[MS_T] = [S_T] - [S_F]$$

$$[MSe_T] = [M_T] - [MS_T]$$

$$[Se_F] = [Se_T] - [MSe_T]$$

Thus, K_{dc} can be determined at any pD for known total concentrations of $CH_3Hg(II)$, mercaptoacetic acid, and the selenol. As indicated in equation 42, K_D is the displacement constant derived for the fully deprotonated forms of the ligands, i.e. $^-SCH_2COO^-$ and RSe^-. K_{dc} can be re-expressed to allow for the calculation of K_d in the following manner:

$$K_{dc} = \frac{[S_F][MSe_T]}{[Se_F][MS_T]}$$

$$= K_d \cdot \frac{\dfrac{[S_F]}{[^-SCH_2COO^-]} \cdot \dfrac{[MSe_T]}{[CH_3HgSeR]}}{\dfrac{[Se_F]}{[RSe^-_F]} \cdot \dfrac{[MS_T]}{[CH_3HgSCH_2COO^-]}}$$

$$= K_d \cdot \frac{\alpha_{Se} \cdot \alpha_{MS}}{\alpha_S \cdot \alpha_{MSe}} \qquad [52]$$

where the α's are the fractional concentrations of the species in equation 42 for which K_d is defined. Rearranging,

$$K_d = K_{dc} \cdot \frac{\alpha_S \cdot \alpha_{MSe}}{\alpha_{Se} \cdot \alpha_{MS}} \qquad [52a]$$

For the purpose of this determination a computer subroutine program was written for use with the non-linear least squares curve-fitting program KINET, where K_d is determined as an unknown parameter. The subroutine is listed in Appendix A.

E. Determination of Displacement Constants for $CH_3Hg(II)$-Selenol-Thiol Reactions

1. The Homologs $HSe(CH_2)_nCOOH$: n = 1, 2, 3

These straight chain homologs have two ionizable groups; the carboxylic acid and the selenohydryl groups. Their acid-base behaviour has already been described in Section B, and their pK_A values are listed in Table 7. To determine the pK'^{D}_A values of the carboxylic acid groups of the $CH_3HgSe(CH_2)_nCO_2D$ complexes, solutions of the 1:1 CH_3Hg-selenol complexes were prepared by direct titration of each selenol with $CH_3Hg(II)$. The chemical shift-pD

titrations of the complexes [$CH_3Hg(II)$-$Se(CH_2)_nCOOD$] were carried out in the manner described in Chapter 2. Typically, ^1H NMR spectra were measured for 15-20 samples taken between pD 2 and 8 and the chemical shift data for the methylene resonances were used to obtain the pK'^{D}_{A} of the carboxylic acid group which is the only ionizable group on the CH_3Hg-complex. Figure 13 shows spectra obtained from the titration of the 1:1 $CH_3Hg(II)$-3-seleno-propanoic acid complex. The spectra show a pair of triplet resonances, which are due to the two pairs of methylene protons of 3-selenopropanoic acid and a singlet resonance from the methyl protons of $CH_3Hg(II)$ in the complex and a singlet resonance from the methyl protons of the chemical shift reference, t-butanol.

Chemical shift data measured as a function of pD were used to determine pK'^{D}_{A} of the carboxylic acid group by fitting the data to equation 21 using KINET. Derivation of the equations used for this calculation can be found in Chapter 2, Section E. The pD dependence of the chemical shift of the methylene protons of the $CH_3Hg(II)$-2-seleno-ethanoic acid complex is shown in Figure 14. The solid curve is the theoretical curve predicted with the acid dissociation constant determined here. Table 8 lists the acid dissociation constants and the limiting chemical shifts for the $CH_3Hg(II)$-selenol complexes for this

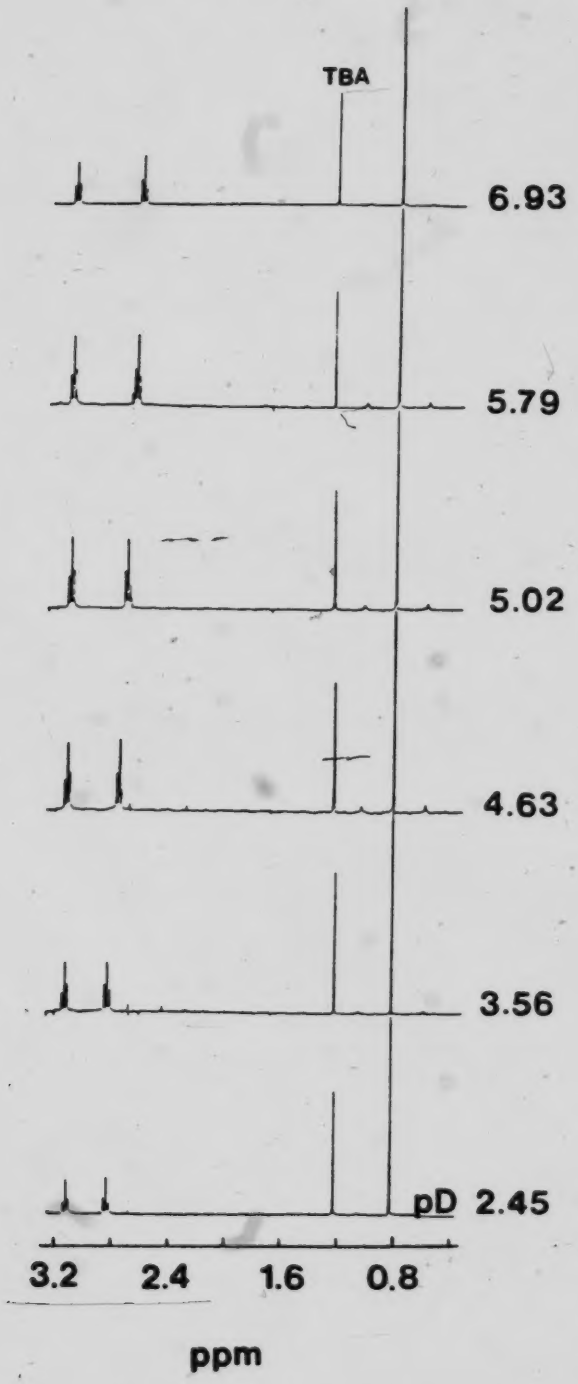


Figure 13. 360 MHz ^{1}H NMR spectra of the complex $CH_3Hg(II)$-3-seleno-propanoic acid at various pD values (17.16_0 mM $CH_3Hg(II)$, 17.09_9 mM RSeH).

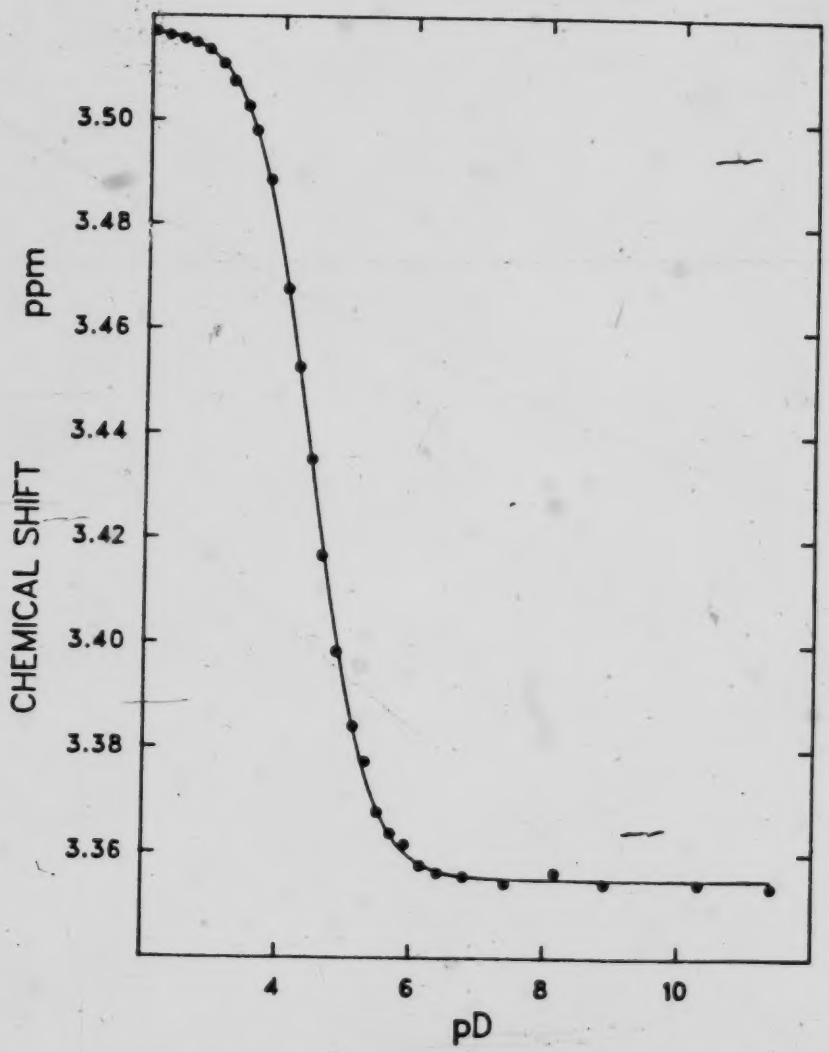


Figure 14. Chemical shift of the methylene protons of the 1:1 $CH_3Hg(II)$-2-selenoethanoic acid as a function of pD (17.05_1 mM $CH_3Hg(II)$, 15.05_2 mM RSeH).

Table 8. pK'^{D}_{A} and limiting chemical shifts of CH_3Hg-selenol complexes for the selenoalkanoic acids.

Complex	Acid Group	pK'^{D}_{A}	δ'_1	δ'_2
$CH_3HgSeCH_2COOD$	COOD	4.45±0.01	3.5175±0.0004	3.3549±0.0003
$CH_3HgSeCH_2CH_2COOD$	COOD	4.77±0.01	2.7838±0.0006	2.6443±0.0006
$CH_3HgSeCH_2CH_2CH_2COOD$	COOD	5.09±0.02	2.5234±0.0007	2.2871±0.0004

homologous series.

To determine the equilibrium constant for the displacement of mercaptoacetic acid by selenol (equation 52a), solutions of exactly known, approximately equimolar concentrations of $CH_3Hg(II)$, mercaptoacetic acid and the selenol were prepared as described in Chapter 2. For the $CH_3Hg(II)$-2-selenoethanoic acid-mercaptoacetate system, 23 samples were withdrawn as the pD was varied from 4 to 12. The chemical shift of the exchange averaged mercaptoacetate resonance was determined. The results are plotted in Figure 15. Chemical shift data for free and $CH_3Hg(II)$ complexed mercaptoacetic acid are also reproduced for comparison.

The chemical shift of the mercaptoacetate methylene protons is close to that of the free thiol at pD's below 4 indicating that at these pD's the $CH_3Hg(II)$ is essentially all complexed by the 2-selenoethanoic acid. As the pD is increased, the chemical shift begins to deviate from that of the free thiol indicating that as the thiol group becomes deprotonated, it begins to compete with the selenol for $CH_3Hg(II)$. The observed chemical shift of the methylene protons of mercaptoacetic acid thus lies between those of the free and the complexed mercaptoacetic acid, the exact shift depending on the extent of the competition. The weaker the $CH_3Hg(II)$-selenol complex,

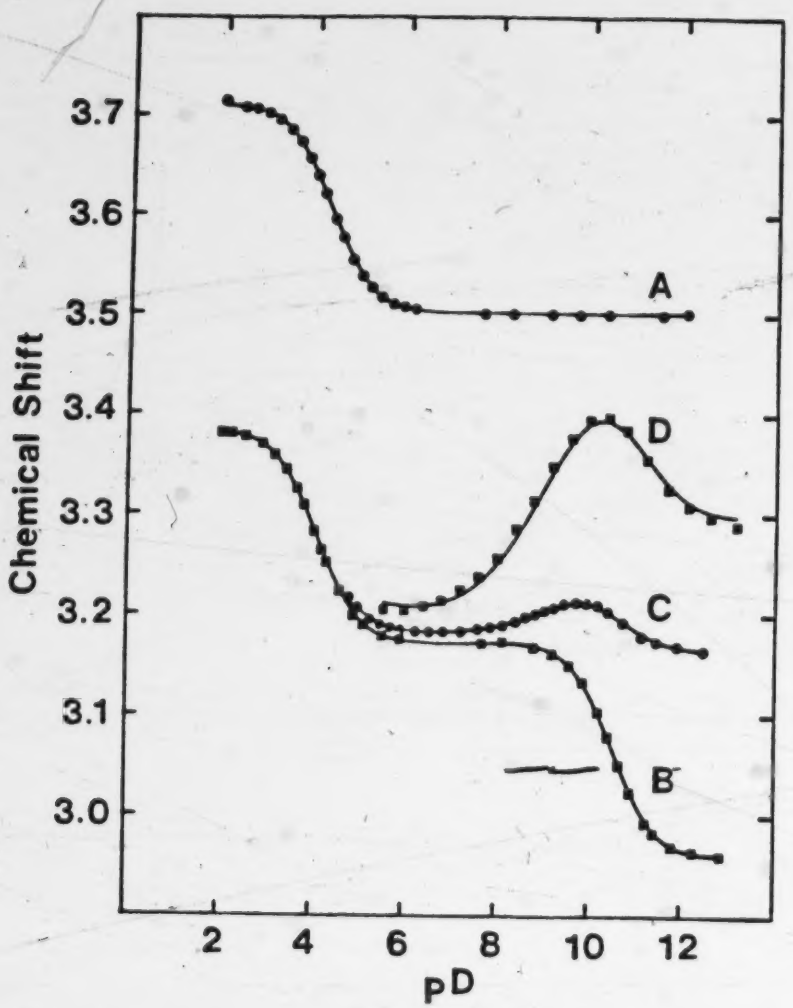


Figure 15. Chemical shift of the methylene resonance of mercaptoacetic acid as a function of pD for

A: free mercaptoacetic acid

B: $CH_3Hg(II)$-complexed mercaptoacetic acid and for solutions containing:

C: 9.00_3 mM $CH_3Hg(II)$, 9.00_3 mM 2-selenoethanoic acid, 8.88_1 mM mercaptoacetic acid

D: 10.14_0 mM $CH_3Hg(II)$, 10.11_0 mM selenocysteamine, 7.13_9 mM mercaptoacetic acid

the poorer it competes for $CH_3Hg(II)$ and the larger the displacement of the chemical shift towards that of the $CH_3Hg(II)$-mercaptoacetate. The observed chemical shift-pD curve can be described by equation 51. The displacement equilibrium constant can therefore be calculated as described in the preceeding section.

A value of log K_d = 0.438 ± 0.004 was obtained by fitting the data to the model described above, with KINET.

Similarly, 13 samples were obtained between pD 8 and 12 for the $CH_3Hg(II)$-3-selenopropanoic acid-mercaptoacetate system. Chemical shift data is shown in Figure 16. From these data, log K_d was determined to be 0.955 ± 0.005.

A solution containing 8.292 mM $CH_3Hg(II)$, 8.292 mM 4-selenobutanoic acid and 10.030 mM mercaptoacetic acid was used for K_d determination. 360 MHz ^1H NMR spectra obtained as the solution was titrated are shown in Figure 17. The chemical shift of the mercaptoacetate methylene protons are plotted as a function of pD in Figure 16. KINET returned a value of 0.221 ± 0.005 for log K_d in the competition reaction of mercaptoacetic acid and 4-selenobutanoic acid for $CH_3Hg(II)$.

2. 2-Hydroxyethane Selenol

A solution of the 1:1 $CH_3Hg(II)$-selenol complex was

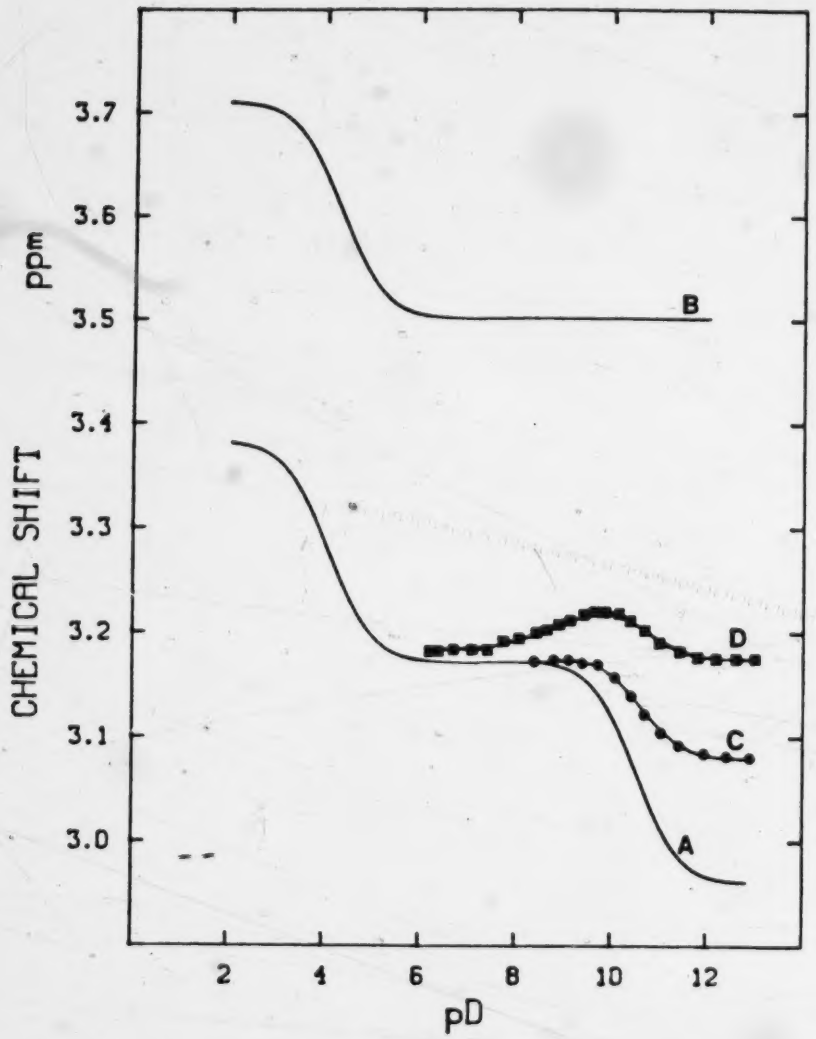


Figure 16. Chemical shift of the methylene resonance of mercaptoacetic acid as a function of pD for

A: free mercaptoacetic acid

B: $CH_3Hg(II)$-complexed mercaptoacetic acid and for solutions containing:

C: 8.58_2 mM $CH_3Hg(II)$, 9.18_3 mM 3-selenopropanoic acid, 9.07_9 mM mercaptoacetic acid

D: 8.29_2 mM $CH_3Hg(II)$, 8.29_2 mM 4-selenobutanoic acid, 10.03_0 mM mercaptoacetic acid.

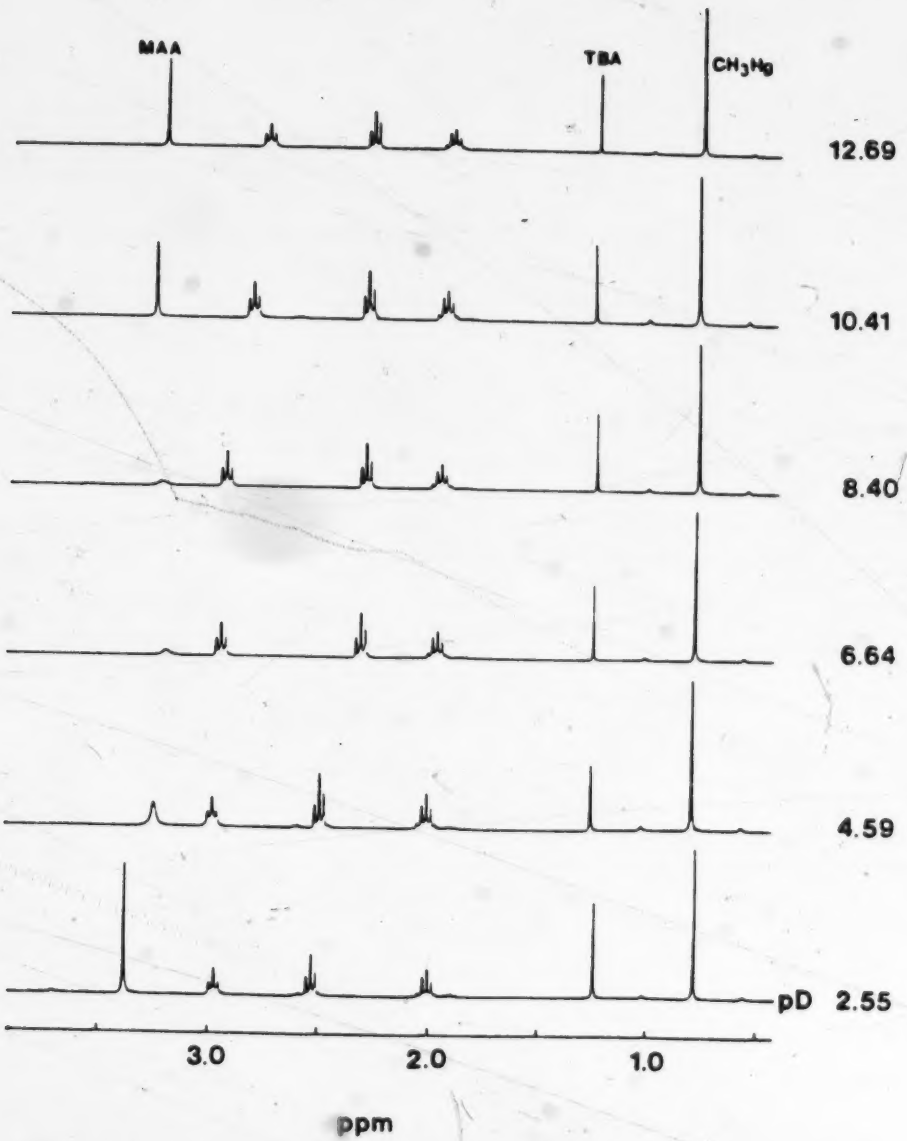
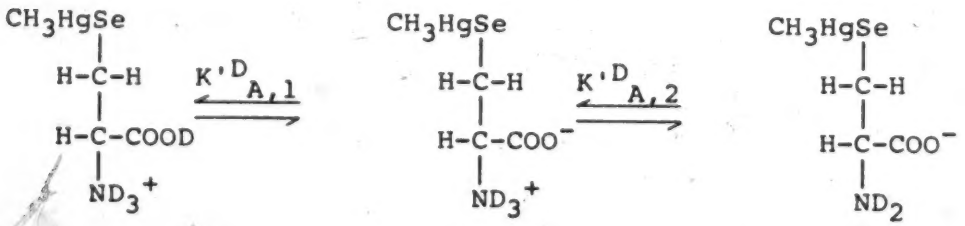


Figure 17. 360 MHz ^{1}H NMR spectra as a function of pD for the thiol-selenol competition reaction for $CH_3Hg(II)$, 8.29_2 mM $CH_3Hg(II)$, 8.29_2 mM 4-selenobutanoic acid, 10.03_0 mM mercaptoacetic acid.

prepared by titrating the selenol with $CH_3Hg(II)$. The solution for the competition reaction was obtained by adding to this solution, a solution of mercaptoacetic acid of known concentration. Twenty samples were withdrawn over the pD range 6 to 12 and their ^1H NMR spectra obtained. The chemical shift data for the mercaptoacetate resonance is plotted in Figure 18. The value obtained for log K_d by fitting the chemical shift data using KINET was -0.714 ± 0.023.

3. Selenocysteine

A solution of the 1:1 $CH_3Hg(II)$-selenocysteine complex was prepared as described in Chapter 2. The $CH_3Hg(II)$-selenocysteine complex has two ionizable protons: the carboxylic acid and the ammonium protons. Equation 53 shows the dissociation scheme for the separate titration of these two groups.



[53]

An ABX type spectrum was observed for the >CH-CH_2

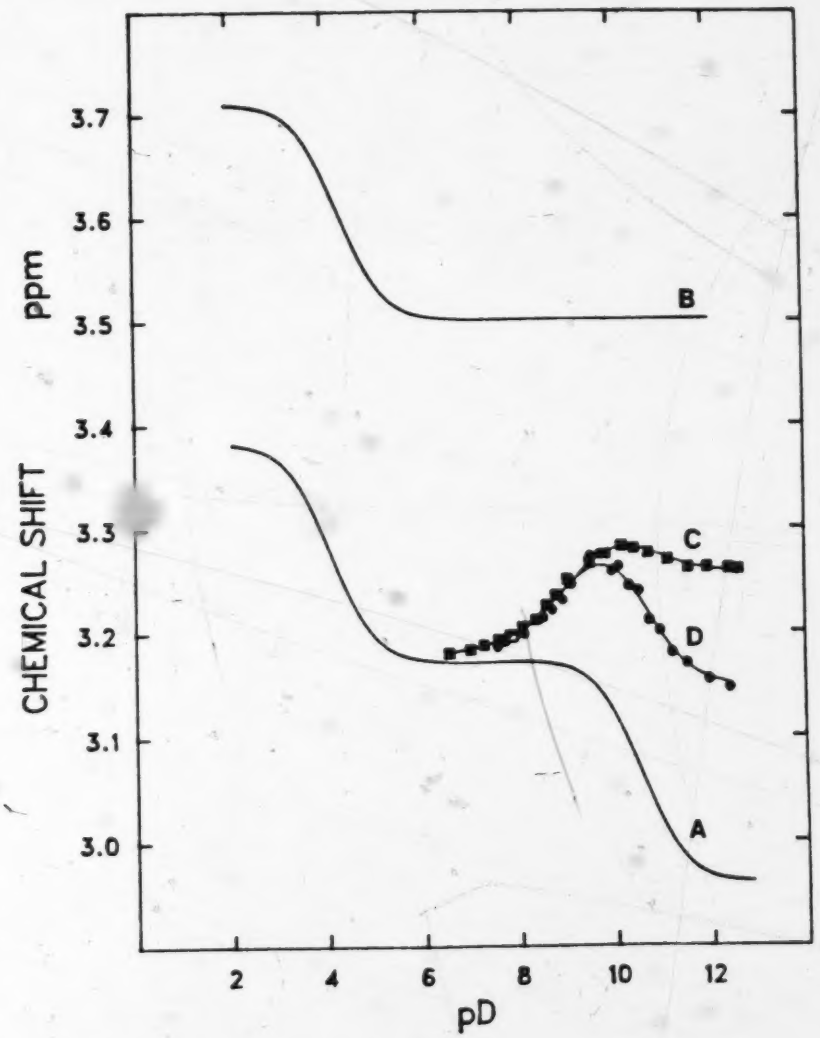


Figure 18. Chemical shift of the methylene resonance of mercaptoacetic acid as a function of pD for

A: free mercaptoacetic acid

B: $CH_3Hg(II)$-complexed mercaptoacetic acid and for solutions containing:

C: 10.65_2 mM $CH_3Hg(II)$, 10.67_0 mM 2-hydroxyethane selenol, 9.61_8 mM mercaptoacetic acid

D: 5.43_3 mM $CH_3Hg(II)$, 5.43_3 mM selenocysteine, 6.12_9 mM mercaptoacetic acid.

protons of selenocysteine. Since the methine proton will be the most sensitive to the titration of the carboxylic acid and ammonium groups, its chemical shift was monitored. The resonance for the methine proton is a three or four line pattern (X part of the ABX pattern) over the pD range of interest. The resonance is located close to the residual HOD resonance which sometimes makes it difficult to observe. The values for the acid dissociation constants for the 1:1 $CH_3Hg(II)$-selenocysteine complex were determined by fitting the observed chemical shift data to the equation

$$\delta_{obsd} = \delta'_1\alpha'_1 + \delta'_2\alpha'_2 + \delta'_3\alpha'_3 \qquad [54]$$

where δ'_1, δ'_2, and δ'_3 are chemical shifts for

$CH_3HgSeCH_2\underset{\underset{ND_3^+}{|}}{C}HCOOD$, $CH_3HgSeCH_2\underset{\underset{ND_3^+}{|}}{C}HCOO^-$, and $CH_3HgSeCH_2\underset{\underset{ND_2}{|}}{C}HCOO^-$,

respectively. The α's are the fraction of the complex present as these species at any particular pD. Section E in Chapter 2 describes in more detail how pK_A values are determined from chemical shift-pD data for a diprotic acid. The results obtained are $pK'^{D}_{A,1} = 2.69 \pm 0.04$ and $pK'^{D}_{A,2} = 9.61 \pm 0.03$, and the chemical shifts are $\delta'_1 = 4.4242 \pm 0.0262$ ppm, $\delta'_2 = 4.0352 \pm 0.0052$ ppm and $\delta'_3 =$

3.4775 ± 0.0084 ppm.

The solution used for the competition reaction was prepared as described in Chapter 2. The chemical shift of the resonance for the methylene protons of mercaptoacetic acid was measured for 20 samples over the pD range 7 to 12 to obtain the displacement constant. The chemical shift of this resonance was plotted as shown in Figure 18. The value of log K_d determined from these data is 0.455 ± 0.014.

4. Selenocysteamine

Figure 19 shows ^1H NMR spectra of the methylene protons of the 1:1 CH_3Hg(II)-selenocysteamine complex between pD 2.4 and 12.4. From the non-linear least square fit of the chemical shift data to equation 24 with KINET, the pK'^{D}_{A} of the amino group was calculated to be 10.40 ± 0.03. The chemical shift of the amino protonated species is 3.2628 ± 0.0024 ppm and the amino deprotonated chemical shift is 2.8352 ± 0.0040 ppm. Chemical shift data for the competition reaction of a solution containing 7.14 mM mercaptoacetic acid, 10.14 mM CH_3Hg(II), and 10.11 mM selenocysteamine were obtained over the pD range 6 to 12, and are plotted in Figure 15. log K_d was calculated to be -0.428 ± 0.014. The theoretical curve calculated from using this value for log K_d is also shown as a solid line.

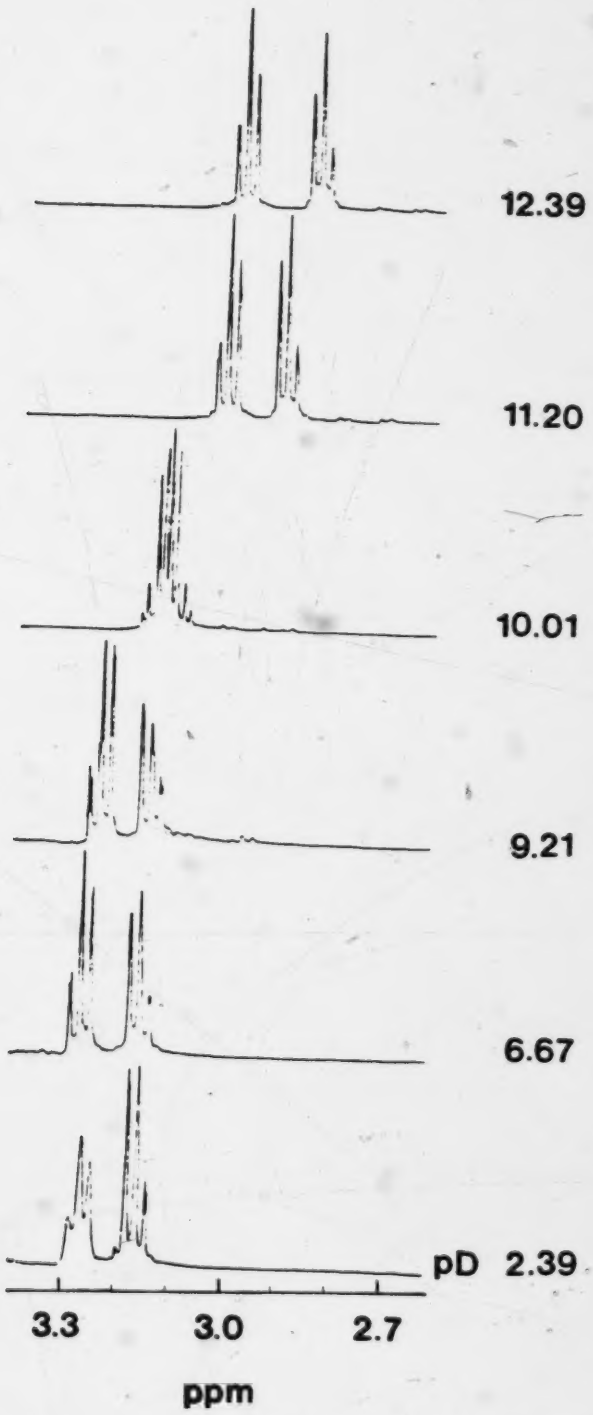


Figure 19. Methylene region of the 360 MHz ^{1}H NMR spectra of the $CH_3Hg(II)$-selenocysteamine complex at various pD values (15.11_4 mM $CH_3Hg(II)$, 15.11_4 RSeH).

5. Selenopenicillamine

The acid dissociation scheme for the 1:1 $CH_3Hg(II)$-selenopenicillamine complex is similar to that shown in equation 53 for the 1:1 $CH_3Hg(II)$-selenocysteine complex. Chemical shift-pD data for the methine proton were obtained over the pD range 2 to 12 for determination of the acid dissociation constants. The data are plotted in Figure 20. From these data, the acid dissociation constants obtained were: $pK'^{D}_{A,1} = 2.53 \pm 0.02$ and $pK'^{D}_{A,2} = 9.41 \pm 0.01$. The chemical shifts of the methine proton in the fully protonated, amino protonated-carboxylic acid deprotonated and fully deprotonated forms of the $CH_3Hg(II)$-selenopenicillamine complex are 4.1147 ± 0.0070 ppm, 3.6569 ± 0.0013 ppm and 3.1677 ± 0.0015 ppm, respectively. Precipitation occurred below pD = 4.4, when a solution containing 12.08 mM $CH_3Hg(II)$ and 12.08 mM selenopenicillamine was titrated. Data were obtained below this pD by halving the concentration.

The displacement constant for the reaction was determined using a solution containing 2.829 mM selenopenicillamine, 2.833 mM $CH_3Hg(II)$, and 6.225 mM MAA. Twenty-three samples were taken as the pD of the reaction system was varied over the range 6 to 12. The mercaptoacetate chemical shift-pD data are plotted in Figure 21. The computer fit of the data gives $\log K_d = 0.478 \pm 0.010$.

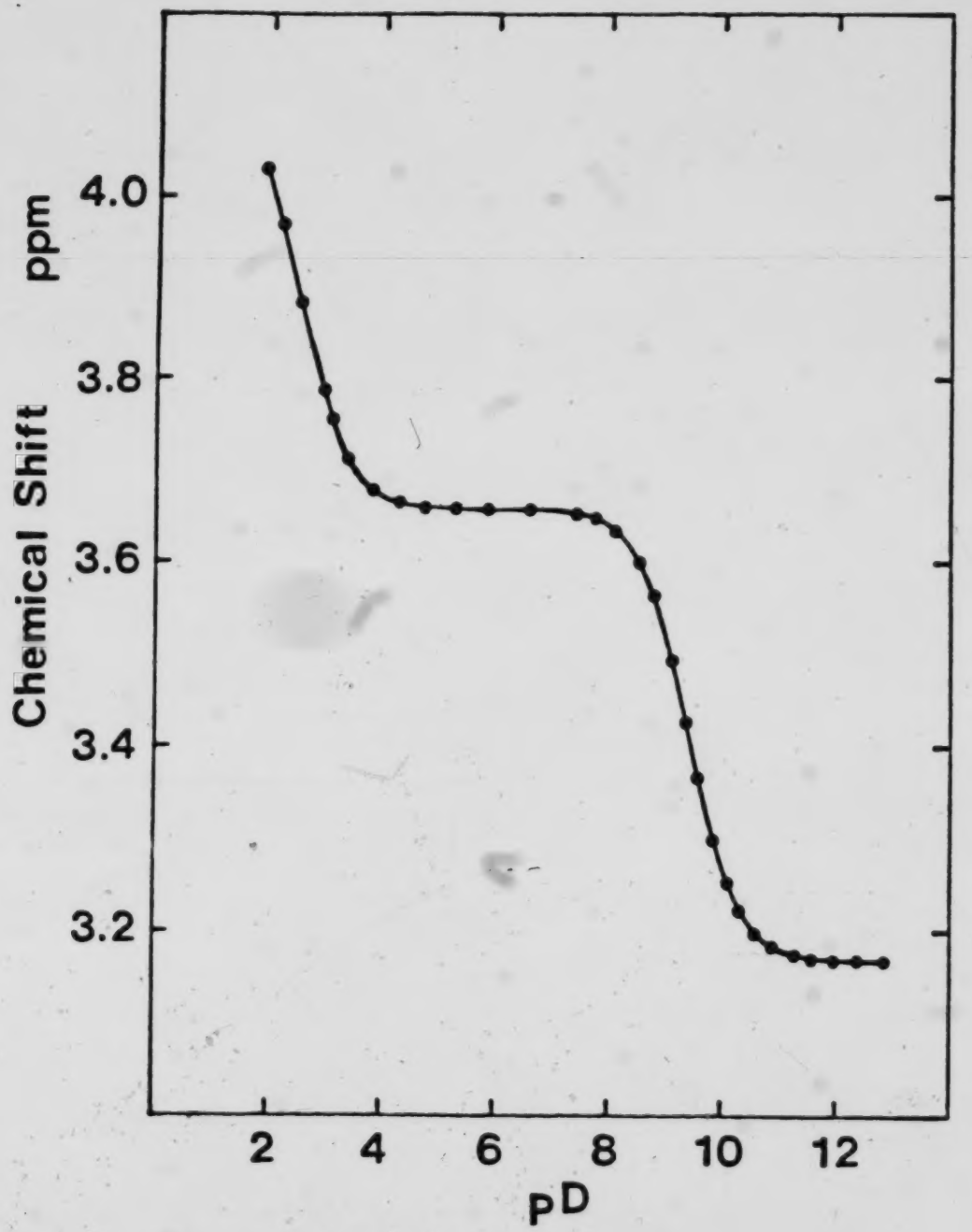


Figure 20. Chemical shift of the methine proton of seleno-penicillamine in the $CH_3Hg(II)$-selenopenicillamine complex as a function of pD (12.08 mM $CH_3Hg(II)$, 12.08 mM RSeH).

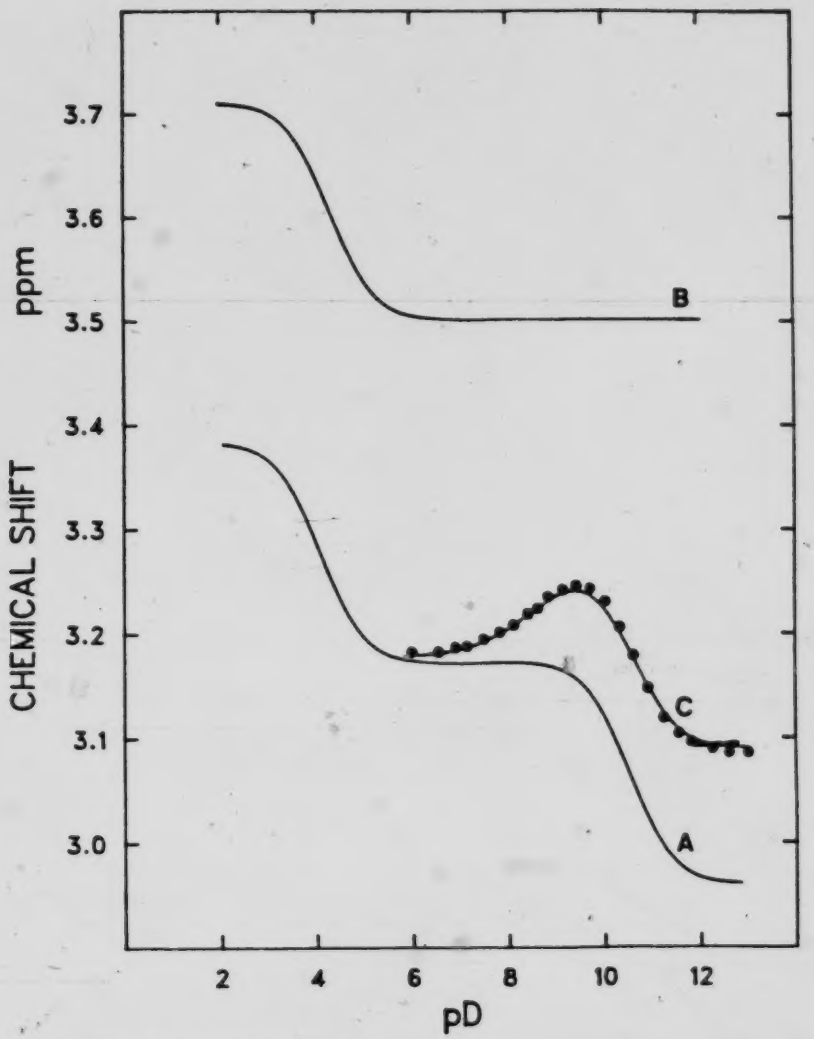


Figure 21. Chemical shift of the methylene resonance of mercaptoacetic acid as a function of pD for

A: free mercaptoacetic acid

B: $CH_3Hg(II)$-complexed mercaptoacetic acid

C: a solution containing 2.83_3 mM $CH_3Hg(II)$, 2.82_9 mM selenopenicillamine, 6.22_5 mM mercaptoacetic acid

F. Determination of Displacement Constants for $CH_3Hg(II)$-Thiol-Mercaptoacetic Acid Reactions

The formation constants for the $CH_3Hg(II)$ complexes of cysteamine, 3-mercaptopropanoic acid, and 4-mercaptobutanoic acid have not been determined previously. In order that a quantitative comparison can be made between the stability of CH_3Hg-Se and CH_3Hg-S complexes, their formation constants were determined in this study. The acid dissociation constants of the ligands were determined by potentiometric titration with data processing by MINIQUAD. The results are listed in Table 9. Unlike the analogous selenoalkanoic acid, the difference in the acidities of the carboxylic acid and thiol groups are sufficiently large that they are titrated separately.

The 1:1 $CH_3Hg(II)$-thiol complexes were prepared in the manner as described earlier for the 1:1 $CH_3Hg(II)$-selenol complexes. The acid-base chemistry of the $CH_3Hg(II)$-thiol complexes was studied by ^1H NMR chemical shift titration. These complexes are monoprotic acids, i.e. either a single carboxylic acid group (on the CH_3Hg-mercaptocarboxylic acid complexes) or a single amino group (on the CH_3Hg-cysteamine complex) is titrated. Table 9 also lists the acid dissociation constants and chemical shifts of the CH_3Hg-thiol complexes.

Table 9. Acid dissociation constants for cysteamine, 3-mercaptopropanoic acid, and 4-mercaptobutanoic acid and their $CH_3Hg(II)$ complexes.

Thiol	$pK^D_{A,1}$	$pK^D_{A,2}$	pK'^D_A
Cysteamine	8.37 ± 0.01	11.12 ± 0.01	9.95 ± 0.01
3-Mercaptopropanoic Acid	4.40 ± 0.02	10.28 ± 0.02	4.42 ± 0.01
4-Mercaptobutanoic Acid	4.63 ± 0.01	10.28 ± 0.02	4.72 ± 0.01

Chemical shifts for the $CH_3Hg(II)$ complexes of cysteamine, 3-mercaptopropanoic acid and 4-mercaptobutanoic acid.

Complex	δ'_{HL} (ppm)	δ'_L (ppm)
$CH_3HgSCH_2CH_2NH_2$	3.2037 ± 0.0020	2.7884 ± 0.0016
$CH_3HgSCH_2CH_2CO_2H$	2.7362 ± 0.0007	2.5168 ± 0.0005
$CH_3HgSCH_2CH_2CH_2CO_2H$	2.5288 ± 0.0004	2.2922 ± 0.0002

For the determination of displacement constants, solutions containing approximately equimolar $CH_3Hg(II)$, thiol, and mercaptoacetic acid were prepared, by adding to a solution of the 1:1 $CH_3Hg(II)$-thiol complex, a known amount of mercaptoacetic acid. The chemical shift of the resonance for the methylene protons of mercaptoacetic acid was monitored as the pD was varied between 8 and 12. The data obtained for each $CH_3Hg(II)$-thiol-mercaptoacetic acid system are plotted in Figure 22. By fitting these data to equation 51, with the non-linear least squares curve fitting routine, the displacement constants for cysteamine, 3-mercaptopropanoic acid, and 4-mercapto-butanoic acid were obtained. The logarithm of the displacement constants are -0.755 ± 0.008, -0.178 ± 0.008, and -0.353 ± 0.007, respectively.

G. Calculation of Formation Constants

The formation constants for the $CH_3Hg(II)$-selenol and $CH_3Hg(II)$-thiol complexes were calculated from the displacement constants and the formation constant of the $CH_3Hg(II)$-mercaptoacetic acid complex using equation 44. Table 10 lists the logarithm of the formation constants for the $CH_3Hg(II)$ complexes of the fully deprotonated ligands.

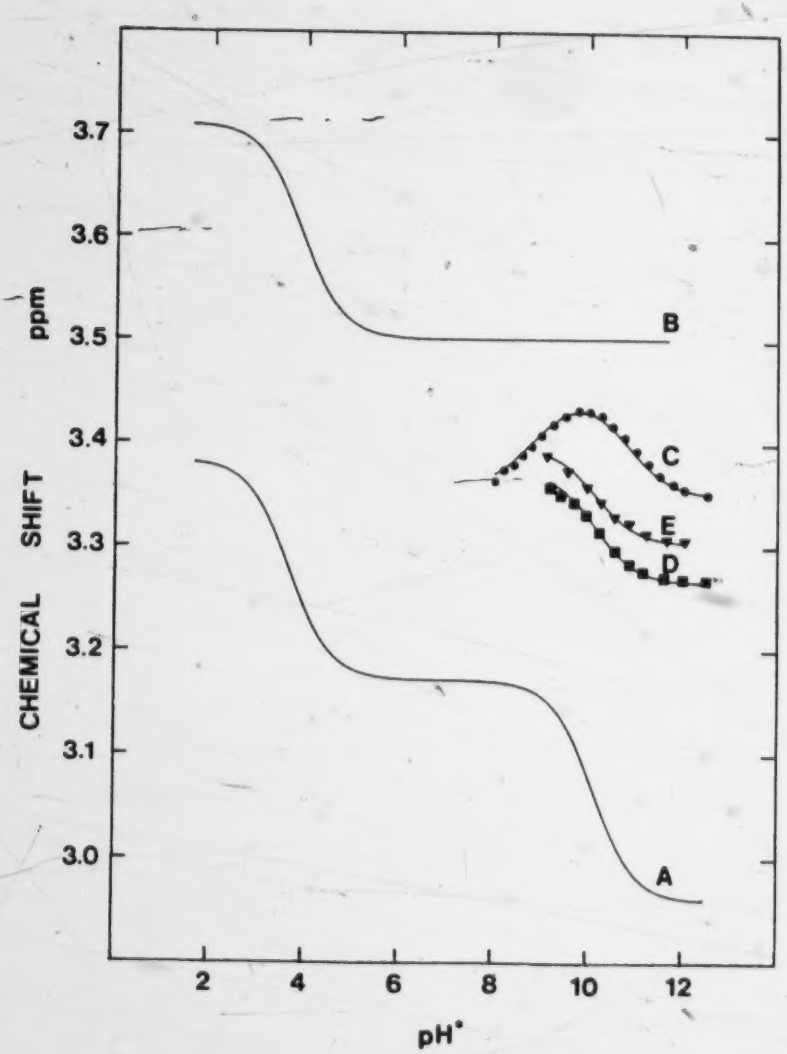


Figure 22. Chemical shift of the methylene resonance of mercaptoacetic acid as a function of pH* for

A: free mercaptoacetic acid

B: $CH_3Hg(II)$-complexed mercaptoacetic acid and for solutions containing:

C: 9.76_7 mM $CH_3Hg(II)$, 9.71_8 mM cysteamine, 9.34_6 mM mercaptoacetic acid

D: 9.84_5 mM $CH_3Hg(II)$, 9.79_7 mM 3-mercaptopropanoic acid, 9.42_1 mM mercaptoacetic acid

E: 10.27_2 mM $CH_3Hg(II)$, 10.26_7 mM 4-mercaptobutanoic acid, 9.15_4 mM mercaptoacetic acid.

Table 10. Logarithm of formation constants of $CH_3Hg(II)$-selenol and thiol complexes.

Complex	log K_f[a] X = Se	X = S
$CH_3HgXCH_2COO^-$	17.36	16.92[b]
$CH_3HgXCH_2CH_2COO^-$	17.88	16.74
$CH_3HgXCH_2CH_2CH_2COO^-$	17.14	16.57
$CH_3HgXCH_2CH_2NH_2$	16.49	16.17
$CH_3HgXCH_2\underset{\displaystyle NH_2}{\underset{\vert}{C}}HCOO^-$	17.38	16.67[b]
$CH_3HgXC(CH_3)_2\underset{\displaystyle NH_2}{\underset{\vert}{C}}HCOO^-$	17.40	16.94[b]
$CH_3HgXCH_2CH_2OH$	16.21	16.12[c]

[a] In D_2O at 25°C and μ = 0.3 M.

[b] In H_2O at 25°C and μ = 0.3 M

[c] Reference 127.

H. Discussion

A direct comparison of the acid dissociation constants for the protonated form of selenocysteine in Table 7 with those for cysteine (pK_{SH} = 8.38 and $pK_{NH_3^+}$ = 8.733) (146) indicates that while deprotonation of the selenohydryl and amino groups of the selenoamino acid do not overlap, deprotonation of the sulphydryl and amino groups occurs simultaneously. The acid dissociation scheme for selenocysteine and cysteine are shown in Figure 23. In the case of cysteine, the scheme can only be completely described with the aid of microscopic acid dissociation constants. The complexity of the latter dissociation scheme can better be appreciated from the species distribution curves as shown in Figure 24. The various protonated species are labelled in correspondence in Figures 23 and 24.

As the electronegativities of selenium and sulphur are similar (165,166), this cannot be used to explain the large differences in the pK_A values of analogous selenols and thiols. The lower pK_A value of the selenohydryl proton as compared to that of the sulphydryl proton can probably be explained on the basis of the hard and soft acid classification of Pearson (152,167). Let the

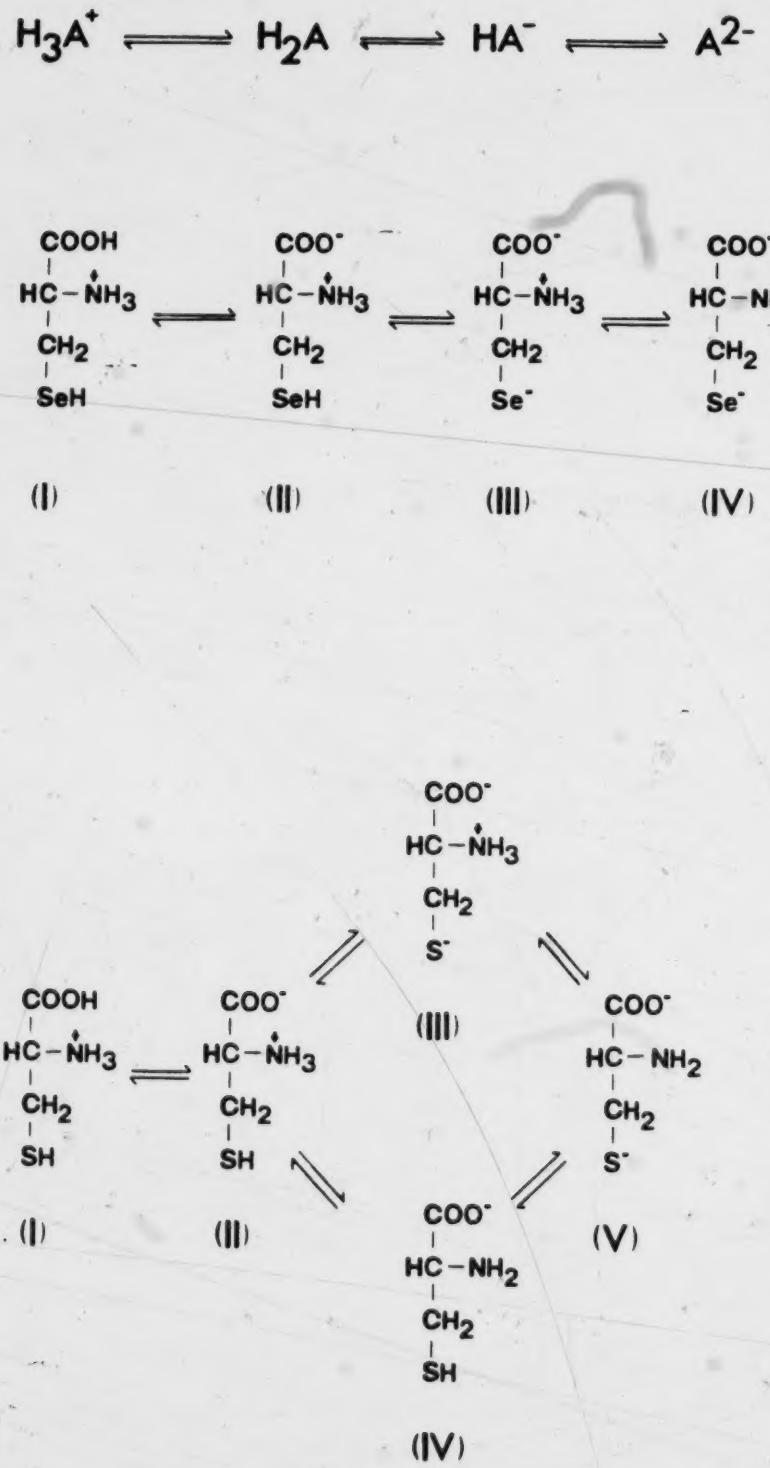


Figure 23. Acid dissociation scheme for A: selenocysteine and B: cysteine.

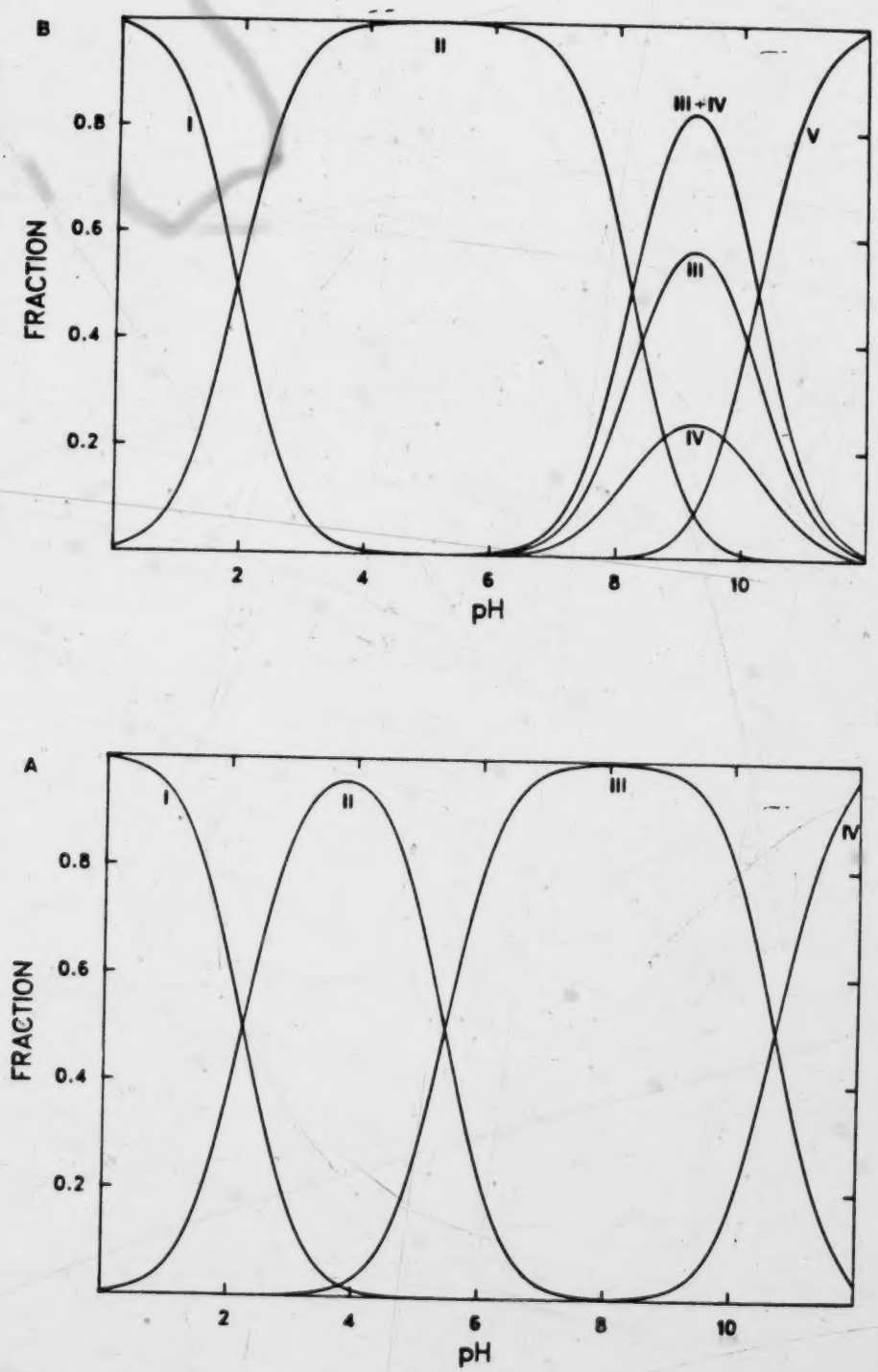


Figure 24. Distribution of protonated and deprotonated species for A: selenocysteine and B: cysteine.

protonation processes be written as

$$RSe^- + H_3O^+ \rightleftarrows RSeH + H_2O \qquad [55]$$

and

$$RS^- + H_3O^+ \rightleftarrows RSH + H_2O \qquad [56]$$

The observed enthalpy change upon protonation of the thiolate and selenolate groups is the sum of all enthalpy changes occurring in the process. This includes the enthalpies of solution of all species which are present, the enthalpy of the proton-water bond breaking, and the proton-chalcogen bond formation. The overall enthalpy change is more favourable for the S-H bond formation (bond energy determination indicates that the S-H bond is stronger [bond energy 369.6 kJ] than the Se-H bond [bond energy 281.4 kJ] (168)). The larger selenium atom renders it more polarizable than the smaller sulphur atom. This means a more favourable entropy change for thiolate protonation due to the larger number of water molecules which are liberated from the thiolate anion when protonation occurs. The less solvated selenolate ion has fewer water ligands around it, thus giving a lower entropy change upon protonation. The overall free energy change is expressed by the equation

$$\Delta G = \Delta H - T\Delta S \qquad [57]$$

From the thermodynamic point of view, the overall free energy change for the protonation of thiolates is more favourable than for the analogous selenolates. Thus, the dissociation of the sulphydryl proton would be less favourable than that of the selenohydryl proton, i.e. the sulphydryl pK_A is higher than the selenohydryl pK_A.

As has been indicated in Section D, the constant for the displacement of mercaptoacetic acid by the selenolate ligands, K_d, was calculated for the model where only the completely deprotonated species of the ligands are involved. Clearly, from Figure 8, protonated species of the ligands are present, and thus other displacement reactions occur at lower pH. Constants for these other reactions can be calculated from the displacement constants for the fully deprotonated species for any system for which the acid dissociation constants of all the species involved are known. For instance, consider the reactions for the triprotic ligand selenocysteine:

$$CH_3Hg(II) + {}^{-}SeCH_2\underset{NH_2}{\underset{|}{C}}HCO_2^{-} \overset{K_f}{\rightleftharpoons} CH_3HgSeCH_2\underset{NH_2}{\underset{|}{C}}HCO_2^{-} \qquad [58]$$

$$CH_3Hg(II) + {}^{-}SeCH_2\underset{{}^{+}NH_3}{\overset{|}{C}}HCO_2^{-} \rightleftharpoons CH_3HgSeCH_2\underset{{}^{+}NH_3}{\overset{|}{C}}HCO_2^{-} \qquad [59]$$

$$CH_3Hg(II) + HSeCH_2\underset{{}^{+}NH_3}{\overset{|}{C}}HCO_2^{-} \rightleftharpoons CH_3HgSeCH_2\underset{{}^{+}NH_3}{\overset{|}{C}}HCO_2^{-} + H^{+} \qquad [60]$$

The formation constants, K_{fh1} (for the amino-protonated selenol) and K_{fh2} (for the selenohydryl and amino-protonated selenol) can be calculated from K_f and the acid dissociation constants by the following relations:

$$K_{fh1} = \frac{K_f \cdot K^D_{A,3}}{K'^D_{A,2}} \qquad [61]$$

$$K_{fh2} = \frac{K_f \cdot K^D_{A,3} \cdot K^D_{A,2}}{K'^D_{A,2}} \qquad [62]$$

$K^D_{A,2}$ and $K^D_{A,3}$ are ligand acid dissociation constants, $K'^D_{A,2}$ is the acid dissociation constant for the ammonium group of the 1:1 $CH_3Hg(II)$-selenocysteine complex and K_f is the formation constant of this complex, determined as detailed in the last section. Similar equations can be written for the other monoprotic and diprotic ligands. Table 11 lists the derived formation constants calculated

Table 11. Derived formation constants for $CH_3Hg(II)$-selenol complexes.

Ligand	log K_{fh1}	log K_{fh2}	log K_{fh3}
$HSeCH_2CH_2NH_2$	15.44	9.94	-
$HSeCH_2\underset{\displaystyle NH_2}{\underset{\vert}{C}}HCOOH$	16.31	10.88	11.36
$HSeC(CH_3)_2\underset{\displaystyle NH_2}{\underset{\vert}{C}}HCOOH$	16.22	11.96	11.25

in this manner. Clearly, for the ligands listed, K_{fhl} is much smaller than K_f, i.e. when the amino group is protonated, there is a considerable decrease in the magnitude of the formation constant.

The results of this study provide the first means for quantitative comparison of the relative binding strengths of $CH_3Hg(II)$ with selenols and thiols. Qualitative comparison from $^2J_{^{199}Hg-^1H}$ coupling constants was used previously to determine the relative binding strengths of $CH_3Hg(II)$ complexes. The ^2J values for the $CH_3Hg(II)$-complexes of selenols and thiols are listed in Table 12 for the fully deprotonated ligands. In all cases, thiolate complexes have larger ^2J values than selenolate complexes, indicating a higher affinity of $CH_3Hg(II)$ for selenohydryl ligands compared to sulphydryl ligands. Quantitative comparison of the relative binding strength of $CH_3Hg(II)$-selenol/thiol complexes is provided by the magnitude of the formation constants of these complexes. From Table 10, the formation constants for the CH_3Hg-selenol complexes are 0.1-1.2 log K units larger than those for the corresponding thiol complexes. A correlation between the logarithm of the formation constants of $CH_3Hg(II)$-thiol complexes and the pK_A of the thiol group has been shown (146). Since the acid dissociation constants of the selenoalkanoic acids which

Table 12. $^{2}J_{^{199}Hg-^{1}H}$ coupling constants for $CH_3Hg(II)$ complexes.

Ligand	$^{2}J_{^{199}Hg-^{1}H}$ (Hz) X = Se[a]	X = S[a]
$HXCH_2COOH$	165.3	170.7
$HXCH_2CH_2COOH$	163.1	168.0
$HXCH_2CH_2CH_2COOH$	161.6	167.5
$HXCH_2CH_2NH_2$	163.6	168.5
	162.0[c]	185.7[c]
$HXCH_2CHCOOH$ (NH$_2$)	166.8	174.0[b]
	164.3[c]	177.2[c]
$HXC(CH_3)_2CHCOOH$ (NH$_2$)	165.0	
		~175[c]
$HXCH_2CH_2OH$	164.3	-

[a]This work, μ = 0.3 M at 25°C in D_2O unless specified otherwise.

[b]Rabenstein, D.L. and Fairhurst, M.T. J. Am. Chem. Soc. 1975, 97, 2086.

[c]Reference 154.

have been determined in this work are mixed constants due to the overlap in the deprotonation of the RSeD and the RCOOD groups on these ligands, a quantitative comparison between the formation constants of the $CH_3Hg(II)$ complexes of the selenoalkanoic acid homologs and the analogous thiol ligands cannot be made.

With reference to the equilibria in Figure 8, displacement of mercaptoacetic acid by selenol occurs to some extent over the entire pD range studied. At high pD values, deuteroxide competes with the ligand for $CH_3Hg(II)$, while at low pD's, deuteration competes with $CH_3Hg(II)$ for the ligands. pH dependent conditional formation constants described by the reaction 63 can be used to better describe the complexation equilibria.

$$CH_3Hg_T + L_T \underset{}{\overset{K_{fc}}{\rightleftharpoons}} CH_3HgL_T \qquad [63]$$

The subscript T denotes the total concentration of the species indicated, i.e. L_T comprises all the various protonated and deprotonated free ligand species, CH_3HgL_T includes all the various protonated forms of the 1:1 $CH_3Hg(II)$ complex, and CH_3Hg_T includes all free $CH_3Hg(II)$ species which are not complexed by the ligand L. The effect of pH is shown in Figure 25 where the logarithm of

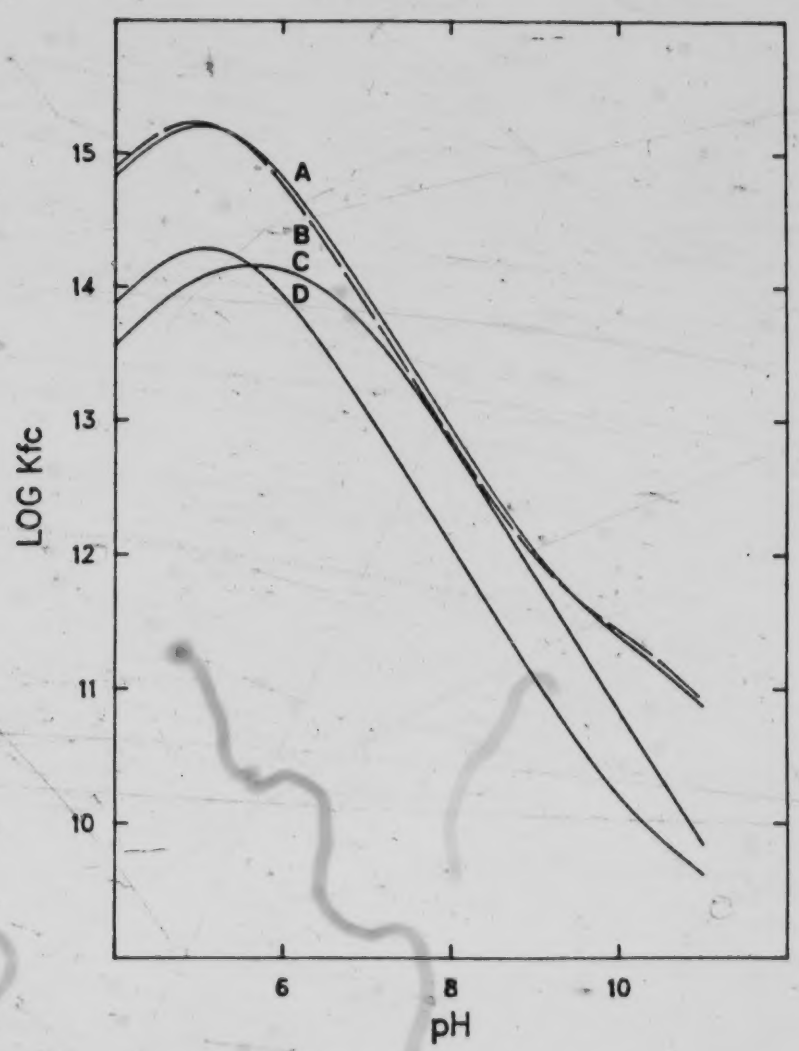


Figure 25. pH dependence of the conditional formation constants for the $CH_3Hg(II)$-selenol complexes of
A: selenocysteine
B: selenopenicillamine
C: selenocysteamine
D: 2-hydroxyethane selenol.

the conditional formation constants of the $CH_3Hg(II)$ complexes of selenocysteine, selenopenicillamine, selenocysteamine, and 2-hydroxyethane selenol are plotted as a function of pH. Over the entire pH range from 4 to 11, the values are less than those given in Table 10. To show the different pH dependence of $CH_3Hg(II)$-thiol and $CH_3Hg(II)$-selenol complexation, the conditional formation constants for the $CH_3Hg(II)$ complexes of cysteine and selenocysteine have been plotted in Figure 26. As pH decreases, the sulphydryl group begins to be protonated, before the selenohydryl group, i.e. protons compete more effectively for RS^- than for RSe^-, since the sulphydryl group is less acidic than the selenohydryl group. With reference to the species distribution curves for these analogous compounds in Figure 24, the thiol group (B, species III and V) is almost completely protonated at pH 6, while a large fraction of the selenohydryl group (A, species III) is still deprotonated. Hence, the difference in the two conditional formation constants increases to a maximum at the selenohydryl pK, as pH decreases. This reflects the increased stability of $CH_3Hg(II)$-selenol complex relative to the $CH_3Hg(II)$-thiol complex, as the pH is decreased.

In the displacement reactions, it was observed that the linewidths of the mercaptoacetate resonances in the $CH_3Hg(II)$-mercaptoacetate-selenol experiments were

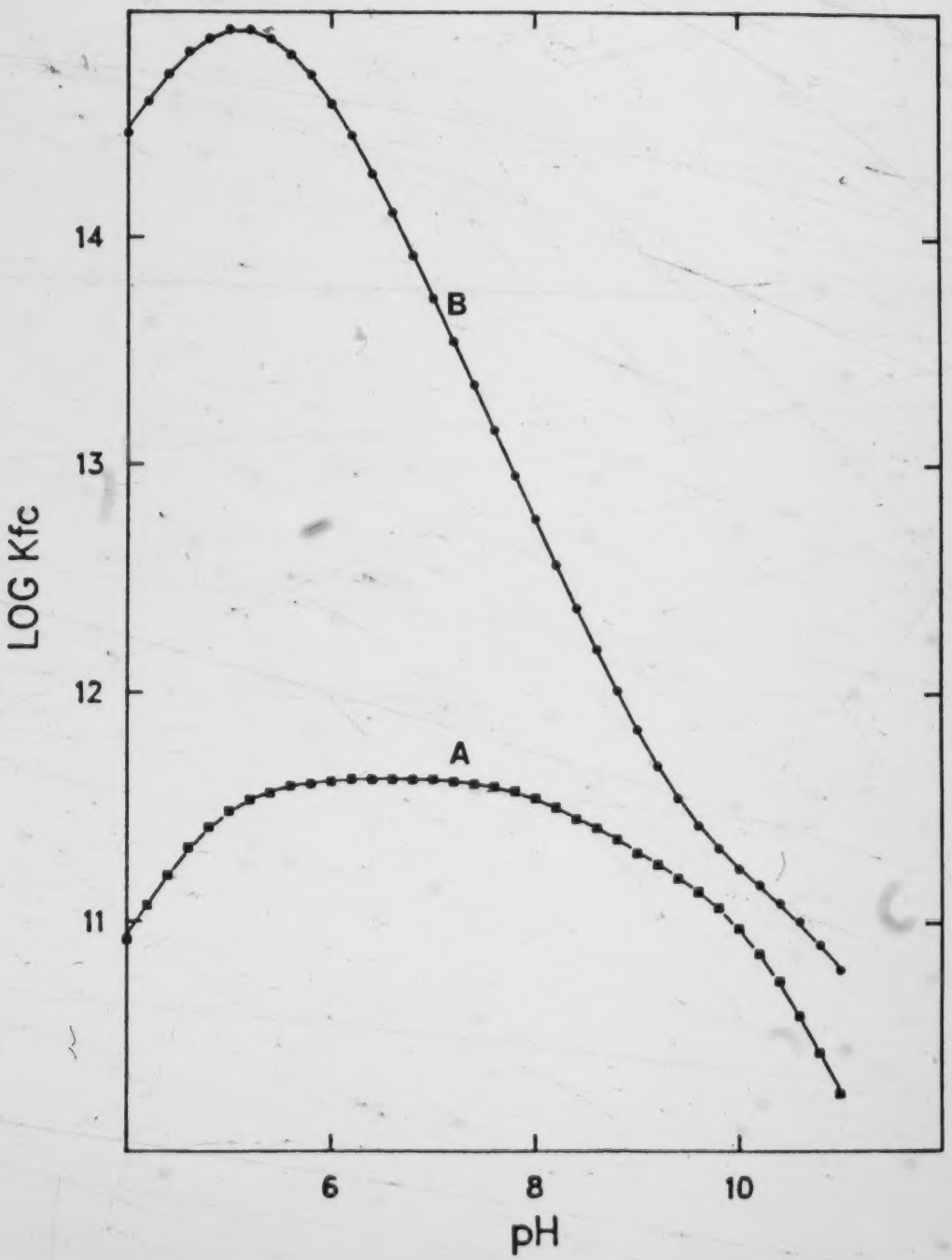


Figure 26. pH dependence of the conditional formation constants for the $CH_3Hg(II)$ complexes of
A: cysteine
B: selenocysteine.

generally narrower than those in the corresponding CH_3Hg-mercaptoacetate-thiol experiments (161). The competition for $CH_3Hg(II)$ by mercaptoacetic acid and a ligand (X = S, Se) can be written as in equation 64:

$$\begin{array}{ccc} RX^- + CH_3HgSCH_2CO_2^- & \rightleftharpoons & CH_3HgXR + {}^-SCH_2CO^- \\ \Big\| & & \\ {}^-S^*CH_2CO_2^- & & \\ \Big\| & & \\ CH_3HgS^*CH_2CO_2^- + {}^-SCH_2CO_2^- & & \end{array} \qquad [64]$$

In this scheme, since the exchange reaction between mercaptoacetate (${}^-SCH_2CO_2^-$) species will be common, whether X = S or X = Se, the narrower linewidths which are observed when the competing ligand is a selenol would then suggest that the reaction between $CH_3Hg(II)$-mercaptoacetic acid and selenol is faster than between $CH_3Hg(II)$-mercaptoacetic acid and thiol. $CH_3Hg(II)$ thiolate and selenolate exchange reactions of the type

$$CH_3HgSR + CH_3Hg^*SeR \rightleftharpoons CH_3Hg^*SR + CH_3HgSeR \qquad [65]$$

have also been observed to be fast on the ^1H NMR time scale but slow on the ^{199}Hg NMR time scale (155).

Possible binding of $CH_3Hg(II)$ by the selenol groups on glutathione peroxidase is of particular interest with reference to $CH_3Hg(II)$ poisoning. The X-ray crystallographic structure reveals four selenium containing active sites in the selenoenzyme. The selenium atoms are located in flat depressions. These serve as possible binding sites for $CH_3Hg(II)$; and from the larger formation constants of selenohydryl ligands, the result could be binding of $CH_3Hg(II)$ to these sites, thereby inactivating the enzyme and leading to a loss of its function to prevent peroxidative damage of the cell membranes.

For an equilibrium distribution of $CH_3Hg(II)$ in human erythrocytes and typical concentrations of 2×10^{-6} M glutathione peroxidase (162), 2×10^{-3} M glutathione and 5×10^{-3} M hemoglobin (159), and the respective logarithms of the conditional formation constants of 13.35, 11.55, and 10.7, it was estimated that 1.6, 7.7, 14, and 47% of the selenocysteine groups on glutathione peroxidase would be complexed by $CH_3Hg(II)$ at total $CH_3Hg(II)$ concentrations of 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, and 5×10^{-5} M. The exchange of $CH_3Hg(II)$ with glutathione and hemoglobin in human erythrocytes has been found to be sufficiently fast for equilibrium to be established (147,160). Since $CH_3Hg(II)$-mercaptoacetate-selenol

exchange is observed to be fast, it will be likely that equilibrium is rapidly established for the binding of $CH_3Hg(II)$ with glutathione peroxidase, glutathione and hemoglobin in red blood cells.

CHAPTER 4

A STUDY OF THE REACTION OF THIOLS WITH DISELENIDES AND SELENOLS WITH DISULPHIDES

A. Introduction

The metabolism of selenium-containing compounds and the mechanism of action of selenium-containing enzymes is thought to involve reaction of the selenium functional groups with thiols and disulphides. This includes the reaction of thiols with diselenides and the reaction of selenols with disulphides. These reactions are described by the forward and reverse reactions in equations 66 and 67.

$$RSH + R'SeSeR' \rightleftharpoons RSSeR' + R'SeH \qquad [66]$$

$$RSH + RSSeR' \rightleftharpoons RSSR + R'SeH \qquad [67]$$

Equations 66 and 67 indicate that the reaction of thiol with diselenide or the reaction of selenol with disulphide is a two step process involving the formation of the mixed selenenyl sulphide, RSSeR'. The overall result of the

reaction of thiol with diselenide is the oxidation of the thiol to the disulphide and the reduction of the diselenide to the selenol.

Although such reactions are thought to be the mechanism by which selenium-containing compounds are metabolized and to be involved in the mechanism of action of glutathione peroxidase, there is surprisingly little known about the details of the reactions. For example, the properties of the intermediate selenenyl sulphides formed in the reaction of diselenide-containing biological molecules with thiols have not been characterized. The most detailed study to date involves the reaction of glutathione (GSH) and cysteine (CySH) with selenocystine (CySeSeCy) (90). From absorbance changes at 248 nm in the uv spectrum, Dickson and Tappel concluded that glutathione and cysteine can completely reduce selenocystine or CySeSR to selenocysteine (CySeH). Equations 66 and 67 describe these reactions. When the thiol is present at concentrations approximately 10^3 times the concentration of the diselenide, as would be expected under physiological conditions (for example, in red blood cells, GSH 2 m$\underline{M}$, RSeSeR 10^{-6} $\underline{M}$), any selenocystine residues, such as CySeSeCy, or selenenyl sulphides, such as GSSeCy and CySSeCy, would be converted to the selenol form.

The above reactions have also been used to

investigate the effect of selenocystine on the activation of sulphydryl enzymes (89); activation usually occurs by thiol-disulphide exchange with cysteine often being used as the activator. In the series of reactions (equations 68-70) proposed for the catalytic effect of selenocystine on papain, a selenol (selenocysteine) reacts with the

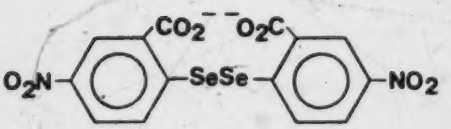
$$CySH + CySeSeCy \rightleftarrows CySSeCy + CySeH \qquad [68]$$

$$CySe^- + H^+ + EnzymeSSCy \rightleftarrows EnzymeSH + CySSeCy \qquad [69]$$

$$CySSeCy + CySH \rightleftarrows CySSCy + CySe^- + H^+ \qquad [70]$$

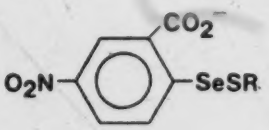
disulphide form of the enzyme in the presence of the activator thiol, cysteine. The selenocysteine is first formed by reaction of cysteine with the selenocystine. It was postulated (89) that this reaction involving the selenoamino acid might occur in biological systems as a mechanism of protection of sulphydryl compounds against oxidation, although other enzymes which are dependent on thiol-containing molecules for activation are inhibited by selenium compounds.

In related studies involving the reaction of 6,6'-diselenobis(3-nitrobenzoic acid), DSNB [IV], with thiols,

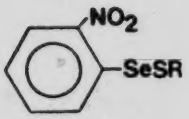


[IV]

it was found that the selenenyl sulphides obtained are more stable than those formed when R and R' of the selenenyl sulphide, RSSeR', are alkyl groups (91,169,170), allowing ^{77}Se NMR spectra to be measured for selenenyl sulphides of the type



where RS is cysteine, N-acetyl cysteine, glutathione, reduced ribonuclease A, or reduced lysozyme. However, other than demonstrating that ^{77}Se NMR spectra can be measured for such compounds, no information was obtained about the formation or the properties of these molecules from the ^{77}Se NMR spectra. Also, although a large number of selenenyl sulphides with the general formula



where R is, for example $-CH_2CHNH_2$ (with CO_2H), $-CH_2CO_2H$, $-CH_2-C_6H_5$, etc. have been synthesized and their ^{77}Se NMR spectra obtained, there is no record regarding their properties.

An analytical investigation of the chromatographic separation of selenium and sulphur amino acids by ion exchange has shown that the Se-Se bond in selenocystine is cleaved in the presence of thiols (171) such as cysteine, 2-mercaptoethanol, and dithioglycol, to yield a mixed sulphur-selenium compound, presumably via the reaction of thiol with diselenide.

Even less is known about the details of the reaction of selenols with disulphides, i.e. the reverse reactions in equations 66 and 67. The reactions are predicted to proceed rapidly (91,172) and Gunther takes advantage of the rapid rate to catalyze the reduction of disulphides (172) by small amounts of diselenide in the presence of the reducing reagent, hypophosphorous acid. No information about the reaction mechanism or properties of the intermediate species was presented.

In view of the importance of the reactions described by equations 66 and 67 in the biological chemistry of selenium, and the importance of the selenenyl sulphide in the proposed mechanism of action of glutathione peroxidase (equations 1-3), the feasibility of characterizing such reactions by NMR has been investigated. Of particular interest are reactions involving low molecular weight selenium-containing biological molecules which, as noted above, would form relatively unstable selenenyl sulphides. As compared to other measurement techniques which might be applied to this problem, NMR has the advantage that measurements can be made directly on the complex reaction mixture, with no separations required. The results of ^{1}H and ^{77}Se NMR studies are presented in this chapter.

B. Selenols, Diselenides, and Selenomethionine

With the objective of characterizing the conditions under which ^{77}Se NMR spectra can be obtained for the selenium-containing molecules in equations 66 and 67, measurements were first made on a series of selenols and diselenides. Both ^{1}H and ^{77}Se NMR measurements were made to determine conditions for obtaining spectra by both techniques.

^1H NMR results are shown in Figure 27 for pH* 4.0 and 12.3 D_2O solutions of 20 mM 2-hydroxyethane selenol in 0.3 M KNO_3 to which varying amounts of 10 mM diselenide were added. The ^1H resonances for the selenol are sharp in the absence of diselenide; however, in the presence of small quantities of the diselenide, these resonances become broad due to selenol-diselenide exchange of the type

$$R^*Se^- + RSeSeR \rightleftarrows R^*SeSeR + RSe^- \qquad [71]$$

In the presence of 10% diselenide, at pH* 4.0, broad resonances which have almost lost their multiplet patterns were observed, as shown in the figure. However, at pH* 12.3, broadening of the multiplet pattern can be observed when 10% diselenide was added, although the multiplet pattern can still be distinguished. At both pH* values, the chemical shifts of the resonances shift towards those of the diselenide as increasing amounts of diselenide are added. Spectra similar to those in Figure 27 were also obtained from samples taken during the course of the electrolytic reduction of diselenides to selenols; when the proportion of diselenide to selenol was higher than for the examples in Figure 27, the resonances were even broader than those shown.

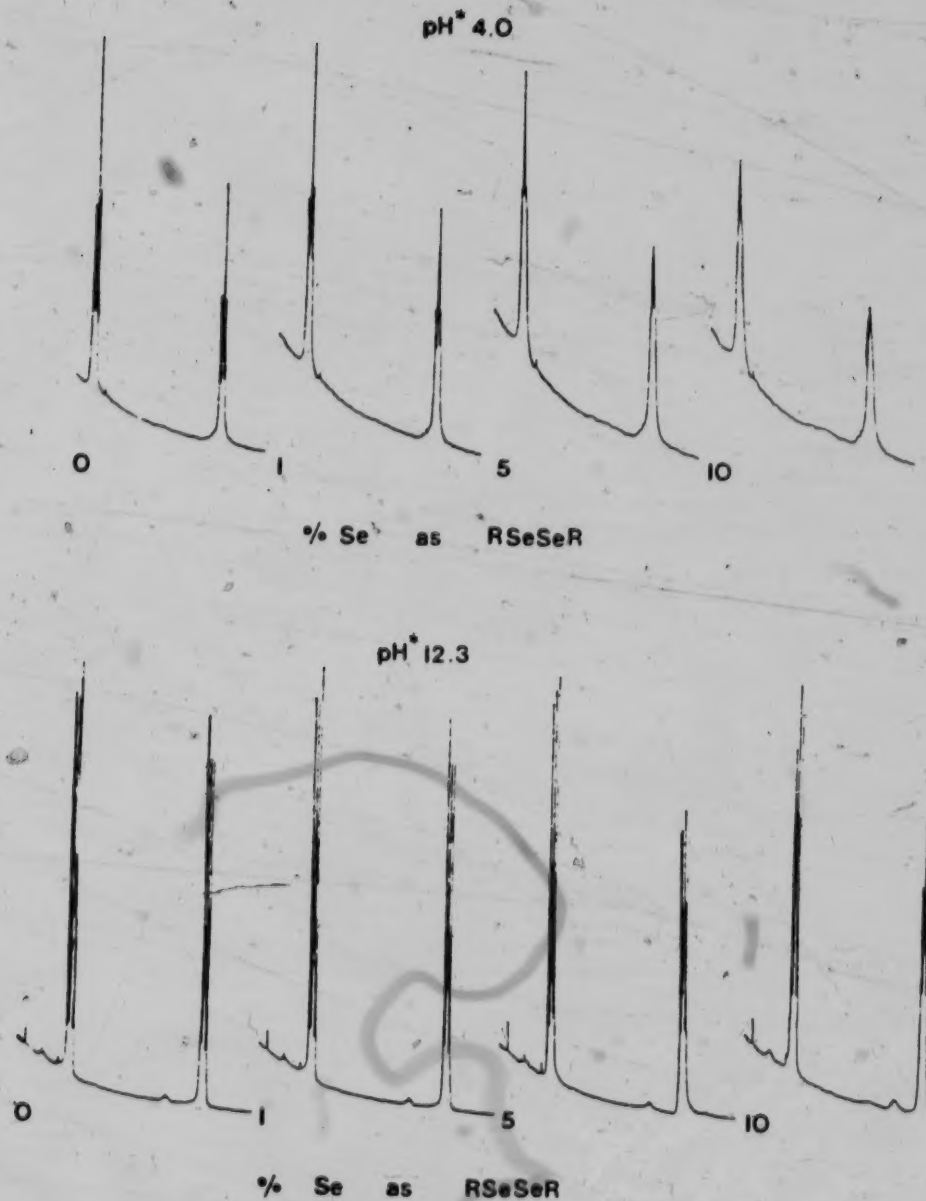


Figure 27. 360 MHz ^{1}H NMR spectra of 2-hydroxyethane selenol at A: pH* 4.0, and B: pH* 12.3, to which varying amounts of bis(2-hydroxyethane)diselenide have been added.

Since exchange broadened resonances are observed in ^{1}H NMR spectra when both selenol and diselenide are present, which would be the case, for example, if they are produced from the reaction of thiol with diselenide or the reaction of selenol with disulphide, it was anticipated that ^{77}Se NMR spectra for such a solution would also exhibit broad resonances. Consequently, in order to obtain sharp resonances in the NMR spectra of selenols, the solution should contain no diselenides and/or selenenyl sulphides; and conversely, for obtaining sharp ^{1}H or ^{77}Se resonances for diselenides and selenenyl sulphides, the solutions should contain no selenols.

In this work, ^{1}H and ^{77}Se NMR spectra were obtained for the diselenodialkanoic acids, $[(HO_2C(CH_2)_nSe)_2$, with n = 1-3], selenocystamine, $[(H_3N^+CH_2CH_2Se)_2]$, and D-,L-selenocystine $[(^-O_2CCHCH_2Se)_2]$. ^{1}H NMR spectra of
$^+NH_3$
selenocystamine and D-,L-selenocystine are complex due to spin-spin splitting, and the spectrum of selenocystine is further complicated due to overlapping resonances from isomeric species, i.e. D-,D-(L-,L-) selenocystine and D-,L-(L-,D-)selenocystine. ^{77}Se NMR spectra were obtained by Fourier transforming free induction decays which have been multiplied with an exponential function for sensitivity enhancement. The ^{77}Se NMR spectra obtained in

this manner for millimolar diselenide solutions show singlet resonances. The ^{77}Se resonances remain singlets over the entire pH* range from 2 to 12, and the chemical shifts are pH* dependent as illustrated by results presented later in this section. The stereoisomers of selenocystine could be resolved as two resonances as illustrated by the ^{77}Se NMR spectrum shown in Figure 28. The ^{77}Se chemical shifts of the diselenides studied in this work were found between 230 ppm and 340 ppm; the ^{77}Se chemical shifts observed for the various diselenides are given in Table 13. The chemical shifts of ^{77}Se resonances for diselenides are reported (109) to lie in the range from 275 ppm to 470 ppm to high frequency from the resonance for $(CH_3)_2Se$.

Spin lattice relaxation times for selected diselenide molecules were estimated by the inversion recovery pulse sequence. The results are listed in Table 14. For comparison, ^{77}Se T_1 measurements made at 19.1 MHz were reported to be longer than 10 seconds (96) for some dialkyldiselenides. The T_1 determined for 0.1 M diselenodiethanoic acid shows that inversion recovery experiments require long experiment times, i.e. more than 12 hours. Hence, to obtain ^{77}Se NMR spectra of diselenides when a repetition time of 1.024 s was used, a smaller flip angle, usually from 10° to 30° was employed.

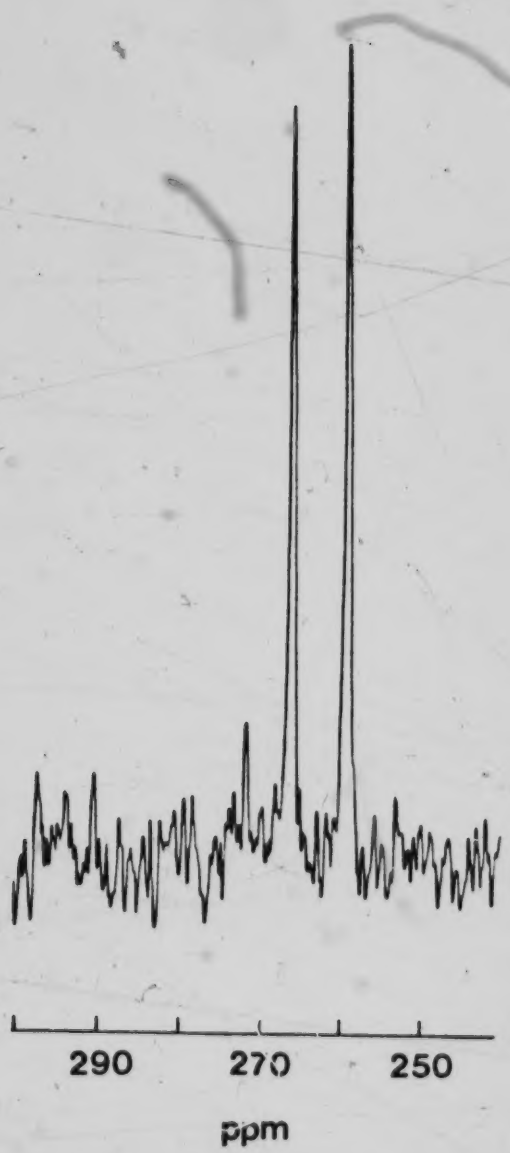


Figure 28. 68.68 MHz ^{77}Se NMR spectrum of a solution of ~30 mM D-,L-selenocystine at pH* 3.65.

Table 13. ^{77}Se NMR chemical shifts of diselenides.

Diselenide	pH* or pD	Chemical Shift (ppm)
Diselenodiethanoic acid	2.09	353.94
	10.00	314.91
Diselenodipropanoic acid	6.61	286.07
Diselenodibutanoic acid	6.99	267.32
Bis(2-hydroxyethane)diselenide	7.80	238.44
Selenocystamine	6.05	251.30
D-,L-selenocystine	6.64	266.35, 259.63
	11.63	254.89, 252.32

Table 14. ^{77}Se T_1 values for selected molecules.

Diselenide	T_1 (s)	pH*
(HO_2CCH_2Se)	7.78 ± 0.54	7.0
Selenocystamine	2.24 ± 0.20	9.38
Selenocystine	1.73 ± 0.33	7.62
	1.04 ± 0.19	
$(HOCH_2CH_2Se)_2$	6.07 ± 0.56	7.80
$(HO_2CCH_2CH_2CH_2Se)_2$	3.43 ± 0.53	7.67

Alkyl Selenenyl Sulphide	T_1 (s)	pH*
$HOCH_2CH_2SeSP$-D	1.81 ± 0.05	1.12
D-,L-CySeSP-D	1.26 ± 0.12	3.63
	1.09 ± 0.18	
D-,L-nPSSeC	2.22 ± 0.29	<5.0

In characterizing the solution chemistry of the diselenides, the use of both ^{1}H and ^{77}Se NMR was considered. The chemical shifts of the diselenides was monitored as pH* was varied, to enable the determination of acid dissociation constants for the acidic groups on these molecules. Figure 29 shows chemical shift-pH* data obtained by ^{1}H and ^{77}Se NMR for a 90% aqueous D_2O solution of 0.0508 M diselenodiethanoic acid in 0.3 M KNO_3. The carboxylic acid groups are titrated over the pH* range for which data are presented. Estimates of the pK_A values were obtained by fitting each data set to equation 30. ^{1}H NMR data were also obtained for 90% aqueous D_2O solutions of 10 mM diselenodipropanoic and diselenodibutanoic acids in 0.3 M KNO_3. Attempts to obtain the corresponding ^{77}Se NMR data were frustrated due to insolubility over the pH* range 2-4, where the molecules are fully protonated. Because of the low concentrations, ^{77}Se NMR spectra obtained over this pH* region have poor signal-to-noise ratios and require long averaging times. The pK_A values for these diselenodialkanoic acids were estimated from ^{1}H NMR data. The results are listed in Table 15.

^{77}Se NMR spectra were measured as a function of pH* for selenocystamine and selenocystine. Chemical shift data for the pH* range 7 to 12 are presented in Figure 30. Over this pH* range, the amino groups are titrated.

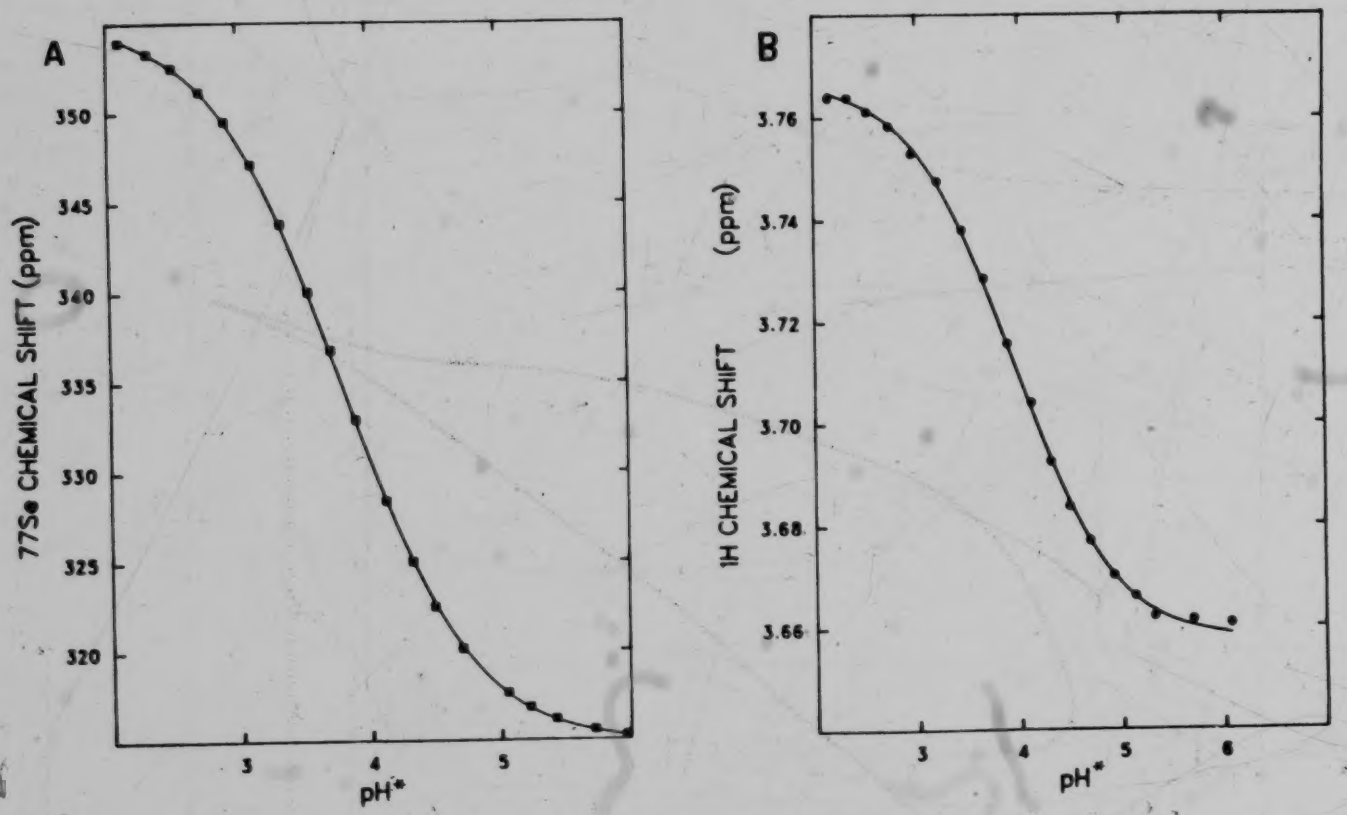


Figure 29. NMR titration curves for a solution of 0.058 M diselenodiethanoic acid
A: ^{77}Se NMR chemical shift as a function of pH*
B: ^{1}H NMR chemical shift of the methylene protons as a function of pH*.

Table 15. Acid dissociation constants and chemical shifts of diselenides.

Diselenide	Acid Group	pK_1	pK_2	δ_{H_2L} (ppm)	δ_{HL} (ppm)	δ_L (ppm)	Nucleus Observed
Diselenodiethanoic acid	COOH	3.47±0.02	4.32±0.03	354.89±0.03	330.77±0.83	314.91±0.01	^{77}Se
		3.49±0.02	4.30±0.01	3.7666±0.0005	3.7167±0.0009	3.6579±0.0005	^{1}H
Diselenodipropanoic acid	COOH	3.88±0.05	4.12±0.04	2.9121±0.0002	2.801±0.0011	2.6781±0.0001	^{1}H
Diselenodibutanoic acid	COOH	4.29±0.08	5.67±0.03	2.1221±0.0007	2.0651±0.0049	1.9333±0.0007	^{1}H
Selenocystamine	NH_3^+	9.65±0.03	10.77±0.11	251.30±0.02	237.75±0.65	234.28±0.03	^{77}Se
D-,L-selenocystine	NH_3^+	8.62±0.04	9.81±0.13	266.35±0.05	257.24±0.50	254.89±0.09	^{77}Se
		8.32±0.15	9.50±0.06	259.63±0.07	257.64±0.42	252.32±0.09	^{77}Se

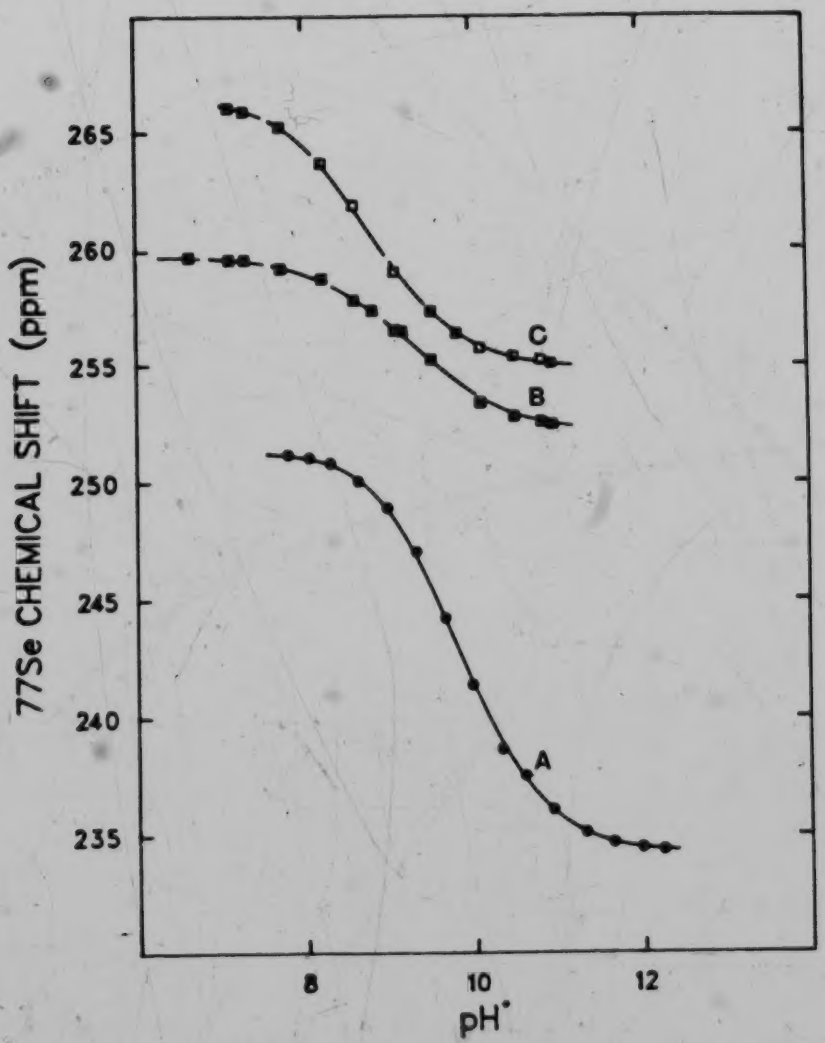


Figure 30. ^{77}Se chemical shift as a function of pH* for
A: 0.047 M solution of selenocystamine
B & C: 0.019 M solution of D-,L-selenocystine.

As shown in Figure 28, two resonances for selenocystine are observed due to the presence of stereoisomeric species. Although the ^{77}Se resonances for D-,L-selenocystine were not assigned to the respective stereoisomers, the variation of chemical shift with pH* for these species could be monitored separately, as illustrated by Curves B and C in Figure 30. Herein is a technique which can be used to study the solution chemistry, in particular the acid-base chemistry, of two stereoisomers in concert. With most analytical techniques this would be difficult and separated pure stereoisomer samples would be required. The pK_A values for the amino groups were calculated from these chemical shift data; the results are listed in Table 15. The acid-base chemistry of the carboxyl groups of selenocystine could not be determined due to the insolubility of selenocystine.

A comparison of the pK_A values obtained for diselenodiethanoic acid by ^1H and ^{77}Se NMR shows that both measurement techniques give results that agree to within the experimental error limits. Thus, ^{77}Se NMR can be used to probe the properties of selenium-containing compounds with accuracy comparable to that of results obtained by ^1H NMR.

The sensitivity of ^{77}Se NMR spectroscopy for characterizing solution chemistry is further illustrated

with selenomethionine. The ^{1}H decoupled ^{77}Se NMR spectra of a 50 mM D_2O solution of selenomethionine $[CH_3Se(CH_2)_2CH(NH_3^+)CO_2^-]$ was observed over the pD range from 3.92 to 11.63. A singlet ^{77}Se resonance was observed and the variation of chemical shift with pD was monitored. Although the selenium is four bonds away from the site of protonation of the amino group, a chemical shift titration curve as shown in Figure 31 was obtained. A value of 9.46 ± 0.03 was obtained for pK^{D}_{A} of the amino group on selenomethionine from these data with the non-linear least squares curve-fitting program KINET. The chemical shift obtained for the amino-protonated, carboxylate-deprotonated species is δ_{HL} = 50.53 ± 0.04 ppm, while that of the fully deprotonated species is δ_{L} = 45.15 ± 0.05 ppm. That ^{77}Se NMR spectroscopy can provide information about groups as far removed as the ammonium group is from the selenium, in selenomethionine further demonstrates the sensitivity of this measurement technique for the studies which have been carried out in this work.

C. Selenenyl Sulphides

Solutions containing selenenyl sulphides, RSSeR', were prepared in either one of two ways: (a) by reaction

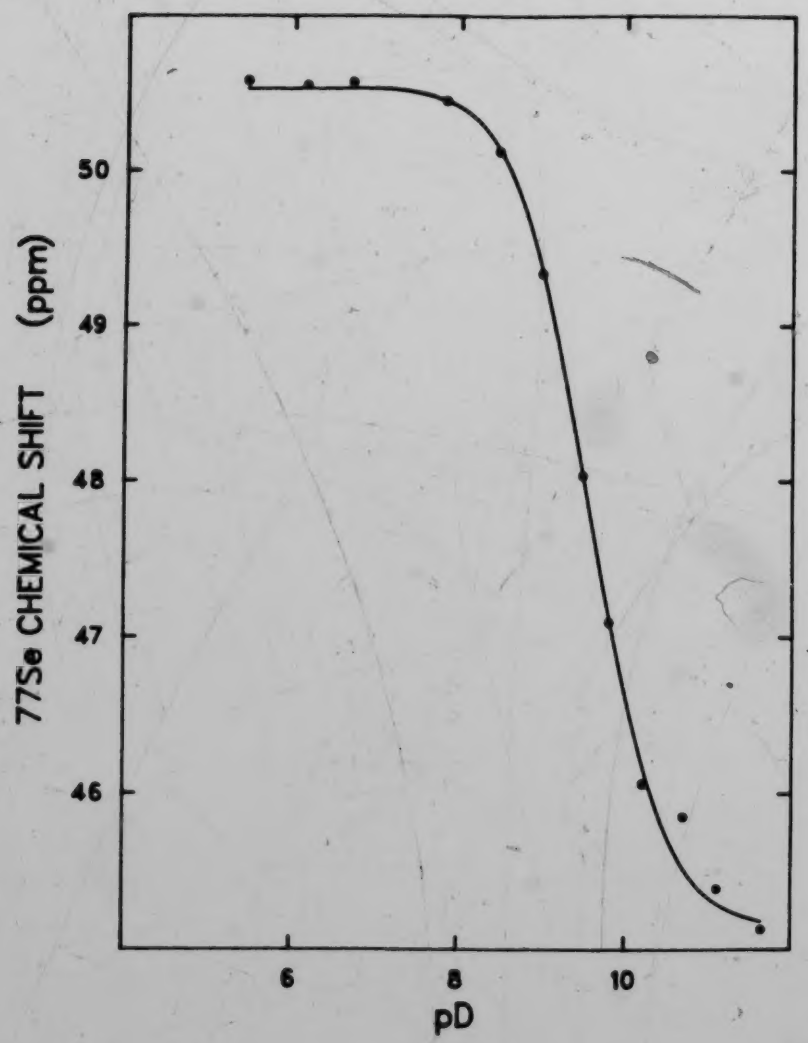


Figure 31. ^{77}Se chemical shift of a 50 mM solution of selenomethionine as a function of pD.

of thiol with diselenide at pH* 9.0 or (b) by chemical reduction of diselenide with sodium borohydride to give selenol and then adding disulphide to this solution. The diselenides and the sulphur amino acids used to prepare selenenyl sulphides for this study are identified in Figure 32. (The N-acetyl derivative of penicillamine was used in one case; the disulphides of some of the thiols were also used.)

Initial investigations show that NMR spectra with sharp resonances can only be obtained for solutions of diselenides and selenenyl sulphides which do not contain any selenol. Since selenols are formed in equations 66 and 67, the reactions used to prepare selenenyl sulphides in this study, care was taken to eliminate selenols in the solutions used for NMR measurements by: (a) using limiting concentrations of selenium-containing compounds, or (b) aerating the solution after mixing the reactants for at least 4 hours to ensure that any selenols had been oxidized to the diselenide. Solutions containing alkyl selenenyl sulphides have a yellow-orange colour. It was observed that when a solution of thiol was added to a solution of diselenide, reaction did not occur below pH* 7.0, since ^{1}H NMR spectra obtained as a function of time show only resonances due to the thiol and the diselenide. However, the reaction proceeds rapidly above

1. **Selenocystamine (CSeSeC)**

$(^{+}H_3NCH_2CH_2Se)_2$

2. **D-,L-Selenocystine (CySeSeCy)**

$$(^{-}O_2CHCH_2Se)_2$$
$$|$$
$$^{+}NH_3$$

3. **Bis(2-hydroxyethane) Diselenide**

$(HOCH_2CH_2Se)_2$

4. **Cysteamine (CSH)**

$^{+}H_3NCH_2CH_2SH$

5. **L-Cysteine (CySH)**

$$^{-}O_2CCHCH_2SH$$
$$|$$
$$^{+}NH_3$$

6. **D-Penicillamine (D-PSH)**

$$^{-}O_2CCHC(CH_3)_2SH$$
$$|$$
$$^{+}NH_3$$

7. **Glutathione (GSH)**

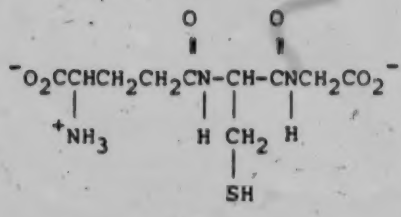


Figure 32. Selenium and sulphur compounds used for preparing alkyl selenenyl sulphides.

pH* 8.0. The reaction of selenol with disulphide, however, proceeds at a faster rate compared to analogous thiol-disulphide exchange reactions.

1. Results from ^{1}H NMR Measurements

Figure 33 shows 360 MHz ^{1}H NMR spectra for solutions prepared by the reaction of

A: 0.05 M cystamine with 0.10 M selenocysteamine at pH* 8.75.

B: 0.20 M cysteamine with 0.05 M D-,L-selenocystine at pH* 9.73.

C: 0.098 M L-Cysteine with 0.05 M D-,L-selenocystine at pH* 9.39.

Spectrum C was obtained from a solution directly after the 0.1 M L-cysteine was added to 0.05 M selenocystine at pH* 9.39. The region plotted shows the ^{1}H resonances of the methylene and methine protons. Broad resonances due to selenol-selenenyl sulphide and selenol-diselenide exchange effects can be observed. This re-emphasizes the requirement that NMR spectra of solutions containing selenenyl sulphides should be obtained in the absence of selenol, in order that sharp resonances may be observed. Spectra A and B were obtained after oxidation of any selenol present from the reaction of thiol with diselenide

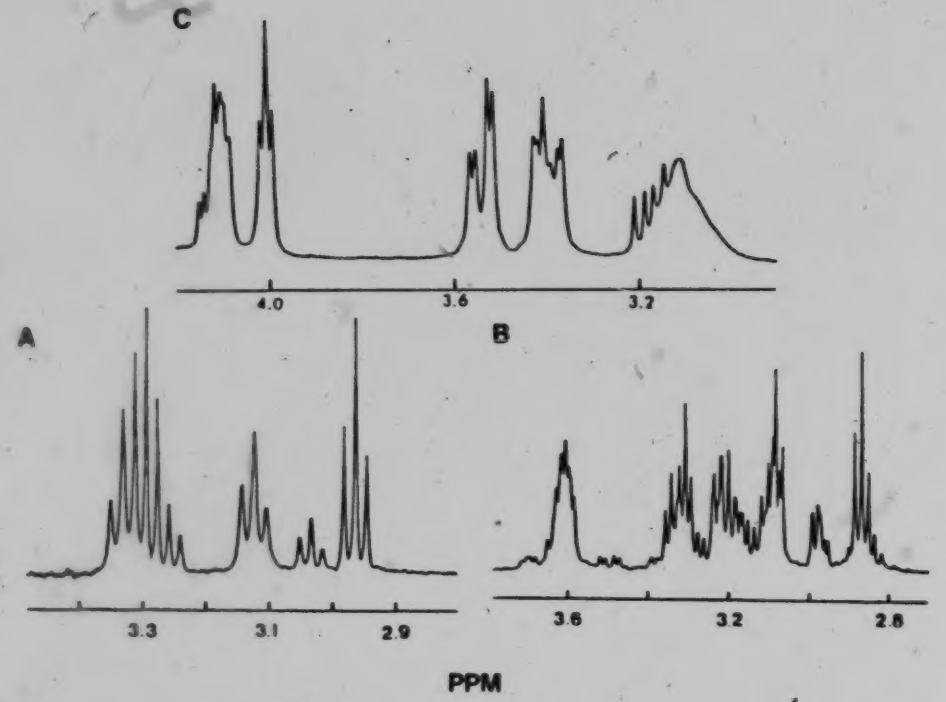


Figure 33. 360 MHz ^{1}H NMR spectra of solutions containing products of the reaction of
A: cystamine (0.05 M) and selenocysteamine (0.10 M), pH* = 8.75
B: cysteamine (0.20 M) and D-,L-selenocystine (0.05 M), pH* = 9.73
C: L-cysteine (0.098 M) and D-,L-selenocystine (0.050 M), pH* = 9.39.

or selenol with disulphide. Resonances from methylene protons are shown in Spectrum A. Four different pairs of triplets were expected from the selenenyl sulphide, disulphide, thiol and diselenide. Three pairs can be distinguished, although one of these appears broader and less sharp. This may be due to overlapping of fairly similar methylene protons. The complexity observed in ^{1}H NMR spectra is further illustrated by Spectrum B which shows considerable overlap and complex spin-spin splitting of the methylene and methine protons of the various isomers present in the reaction mixture. Figure 34 shows ^{1}H NMR spectra of a reaction mixture obtained by reacting 49.80 mM D-penicillamine with 47.35 mM D-,L-selenocystine, at pD 9.4. The spectra were obtained as the pD of the solution was varied. Although the methylene and methine proton regions of the spectra appear complex, the methyl proton region shows relatively simple resonances. From the methine and methyl resonances of the D-penicllamine portion of the molecule, two stereoisomers (D-PSSeCy-D and D-PSSeCy-L) can be identified, and these can be resolved throughout the pD range covered. Figure 35 is an expanded region showing the methyl resonances at corresponding pD values. From the pD dependence of the chemical shift of these resonances, the acid-base behaviour of the selenenyl sulphides can be obtained. The pK^{D}_{A} values for the

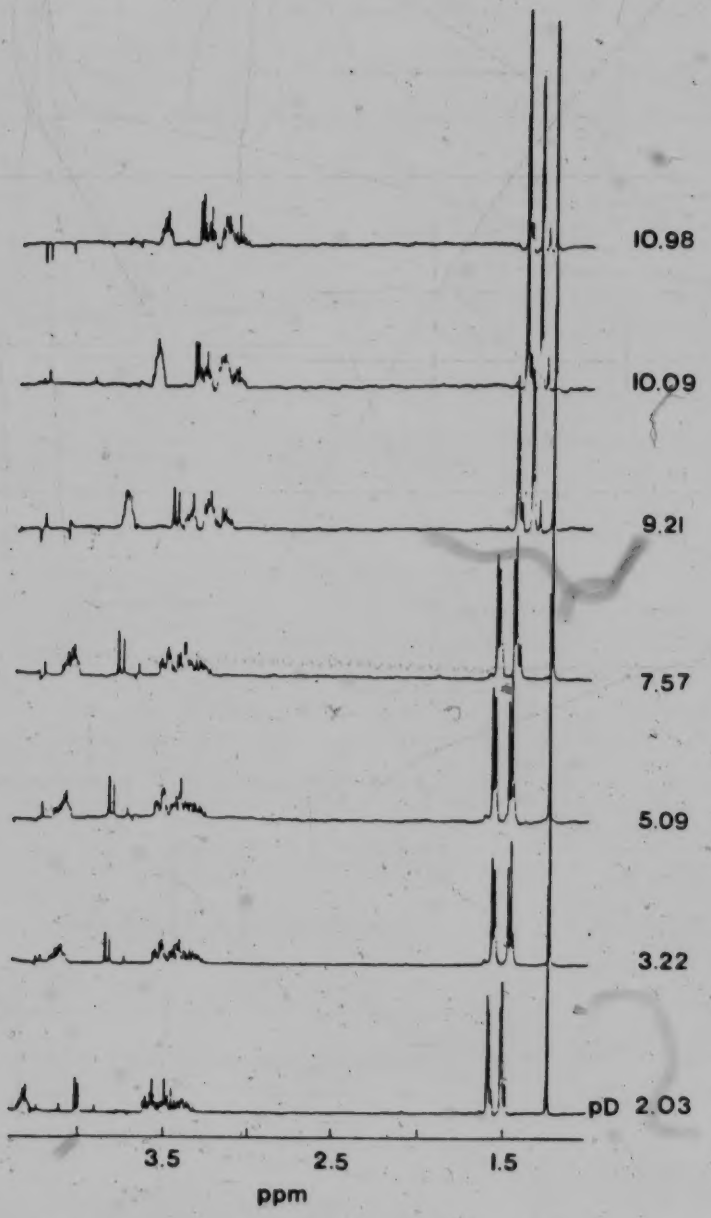


Figure 34. 360 MHz ^{1}H NMR spectra obtained at various pD for the solution obtained by reacting 0.0498 M D-penicillamine with 0.0474 M D-,L-selenocystine at pD 9.4.

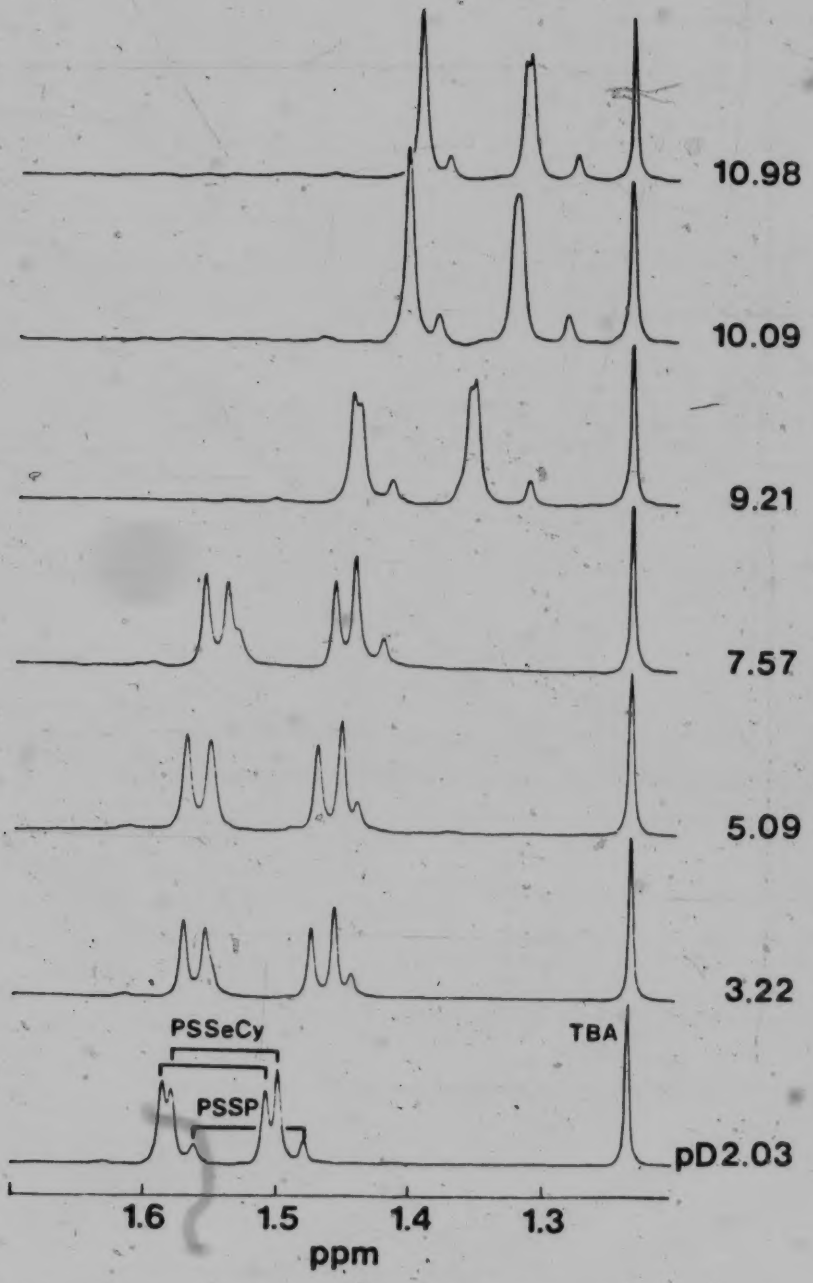


Figure 35. Methyl region of ^{1}H NMR spectra obtained during the titration of a solution prepared from 0.0498 M D-penicillamine and 0.0474 M D-,L-selenocystine at pD 9.4.

ammonium groups have been determined for the isomers from these chemical shift data. The results are given in Table 16.

As evidenced in Figure 33, the ^1H NMR spectra often show overlapping resonances which are difficult to resolve. The selenenyl sulphides containing a D-penicillamine half investigated here have simple resonances in the methyl and/or methine region and their solution chemistry, for example, their acid-base chemistry, can be characterized by monitoring the variation of the chemical shift with pD changes. The selenenyl sulphides characterized in this manner are:

1. $^{-}O_2CCHC(CH_3)_2SSeCH_2CH_2NH_3^{+}$
   $\quad\quad\quad

Table 16. Acid dissociation constants and chemical shifts of selected selenenyl sulphides.[a]

Selenenyl Sulphide	Acid Group	Acid Dissociation Constant	δ_{H_2L} (ppm)	δ_L (ppm)
D-PSSeCH$_2$CH$_2\overset{+}{N}$H$_3$	NH_3^+	pK_2 = 8.65±0.01 pK_3 = 9.96±0.03	1.4688±0.0003	1.3120±0.0004
D-PSSeCH$_2$CO$_2$D[b]	ND_3^+	pK_3 = 9.24±0.01	1.5518±0.0008	1.3897±0.0006
D-PSSeCH$_2$CH$_2$OH	NH_3^+	pK_2 = 9.23±0.00$_2$	3.8269±0.0004	3.3353±0.0004
D-PSSeCH$_2$CH($\overset{+}{N}D_3$)COO$^-$[b,c]	ND_3^+	1. pK_3 = 8.90±0.05 pK_4 = 9.84±0.09	3.8122±0.0012	3.3316±0.0021
		2. pK_3 = 8.98±0.04 pK_4 = 9.85±0.08	3.8374±0.0008	3.3469±0.0015

[a] pK_A values determined in 10% D_2O aqueous solution, μ = 0.3 M.

[b] pK_A value determined in D_2O solution, μ = 0.3 M.

[c] pK_A for the resolved resonances of the two stereoisomers (D-PSSeCy-D and D-PSSeCy-L) are given.

molecule.

2. Results from ^{77}Se NMR Measurements

Solutions of selenenyl sulphides were prepared as described earlier in this section, but at reactant concentrations of ~0.1 M. As ^{77}Se NMR spectra with sharp resonances are difficult to obtain in the presence of selenol, aeration to convert any selenol to the diselenide was carried out for 6 to 8 hours after the thiol had reacted with diselenide for 2 hours. The selenenyl sulphides studied here are relatively unstable due to reaction with other species in the reaction mixture. Equation 67 shows that thiols can react with selenenyl sulphides. It is also possible for selenols to react with the selenenyl sulphides. In the reactions which were used here to prepare selenenyl sulphides, these two reactions contribute to the consumption of selenenyl sulphides; hence, ^{77}Se NMR spectra with good signal-to-noise ratios were difficult to obtain.

^{77}Se spin-lattice relaxation times were evaluated for selected alkyl selenenyl sulphides. The inversion-recovery-pulse sequence was used and the T_1 values were estimated by measuring the resonance intensity as a function of the delay time in the inversion-recovery-pulse sequence. Figure 36 shows spectra measured by the

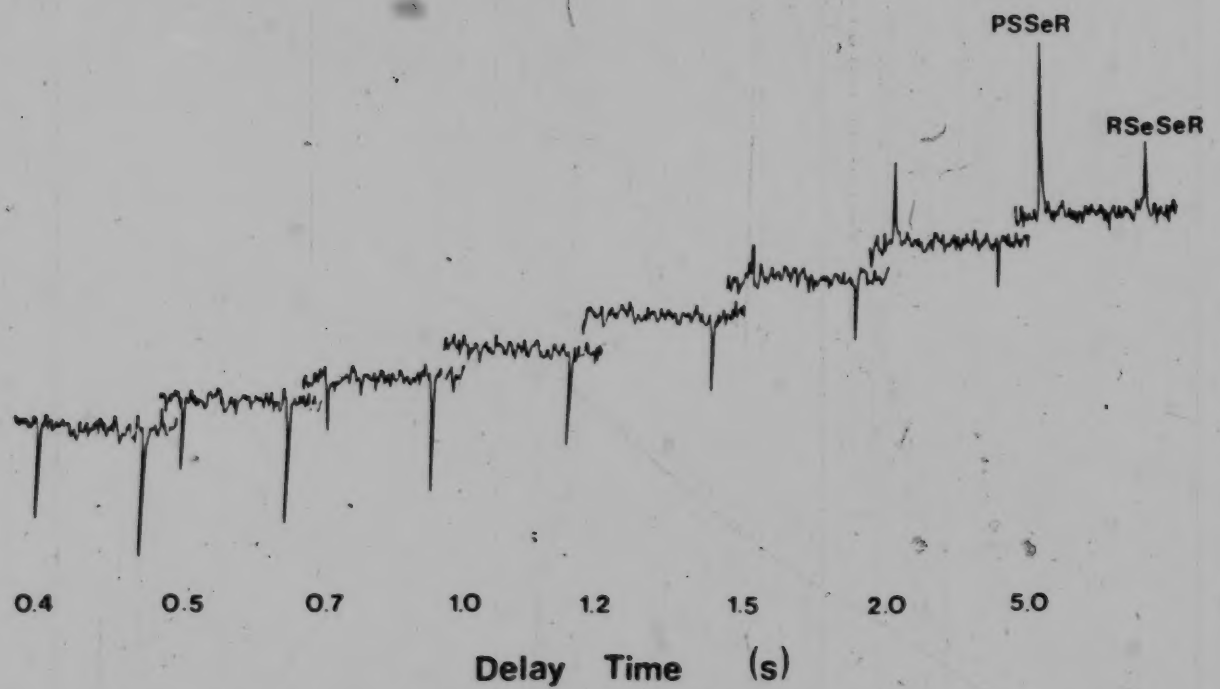


Figure 36. 68.68 MHz ^{77}Se resonances of bis(2-hydroxyethane)diselenide and D-PSSeCH$_2$CH$_2$OH measured by the inversion-recovery-pulse sequence for the determination of spin lattice relaxation times. The times indicated in the figure are the lengths of the delay time in the inversion-recovery-pulse sequence.

inversion-recovery-pulse sequence for a pH* 7.80 solution obtained by reacting 0.2 M D-penicillamine with 0.1 M bis(2-hydroxyethane)diselenide. Because a fairly large number of scans (e.g. 256) were required to obtain spectra having a reasonable signal-to-noise ratio, each experiment utilizes a considerable length of time (e.g. 10.5 hours). Due to the instability of the selenenyl sulphides over this length of time, the values reported in Table 14 have uncertainties up to 15%. Nevertheless, these values do provide an estimate of the magnitude of the spin-lattice relaxation times for ^{77}Se in alkyl selenenyl sulphides. Assuming the T_1 of the ^{77}Se resonance was 1.5 s, ^{77}Se NMR spectra were obtained with repetition times of ~0.2 seconds and a flip angle of ~30° was used. The total experiment time was 60-90 minutes for each ^{77}Se NMR spectrum obtained.

Results from the ^{1}H NMR measurements have indicated highly complicated spectra in many cases. ^{77}Se NMR spectra are simpler than the corresponding ^{1}H NMR spectra, as evidenced by the results in Figures 37 and 38. The spectra in Figure 37 were obtained from the product of the reaction of 0.1 M glutathione with 0.053 M selenocystamine at pH* 9.38. The resonances for the glutamyl methylene protons (2.0 to 2.6 ppm) are broader than the other resonances in the ^{1}H NMR spectrum; this is probably due to

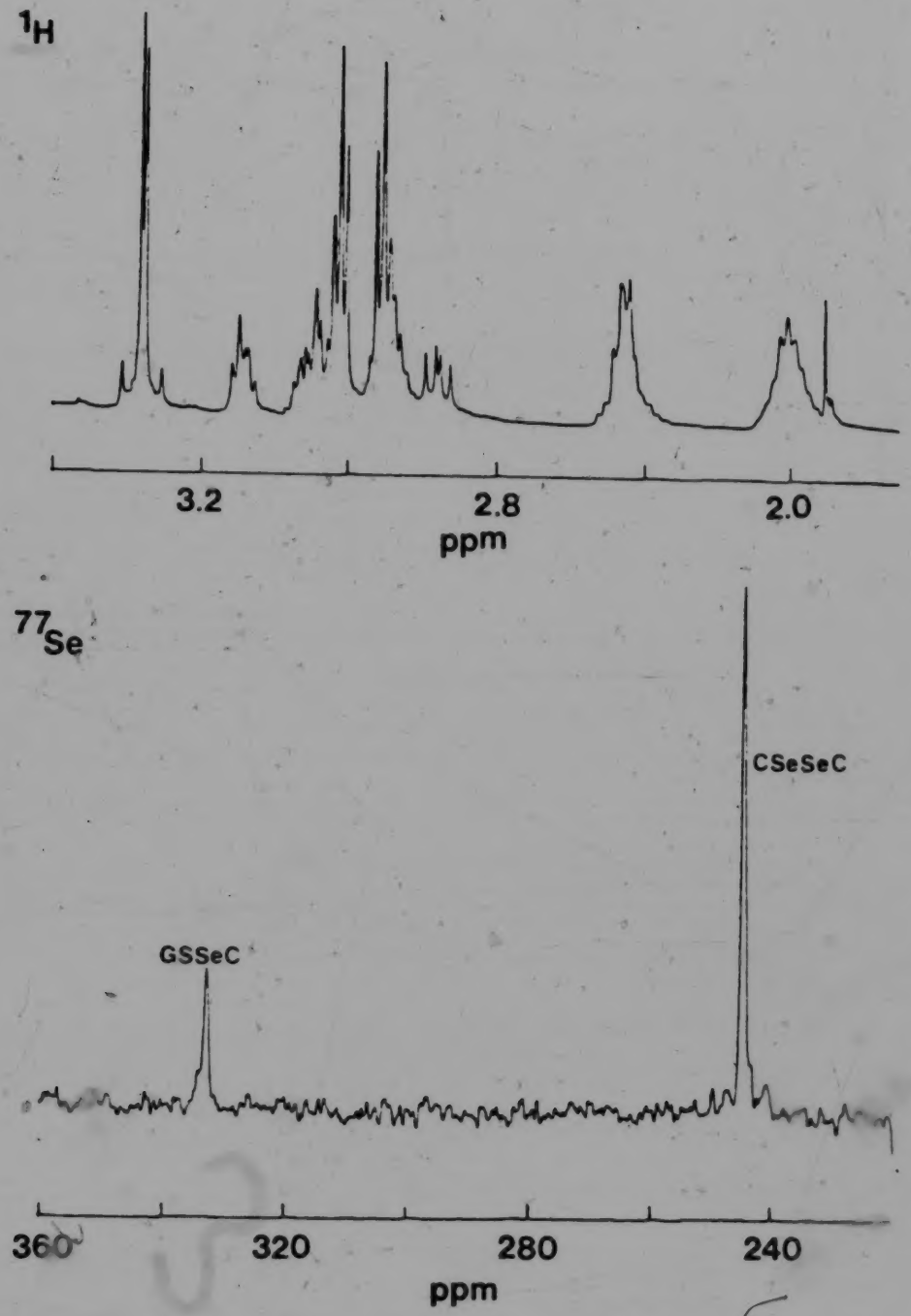


Figure 37. A: 68.68 MHz ^{77}Se NMR and B: 360 MHz ^{1}H NMR spectra of a pH* 3.68 solution prepared by the reaction of 0.10 M glutathione with 0.05 M selenocystamine.

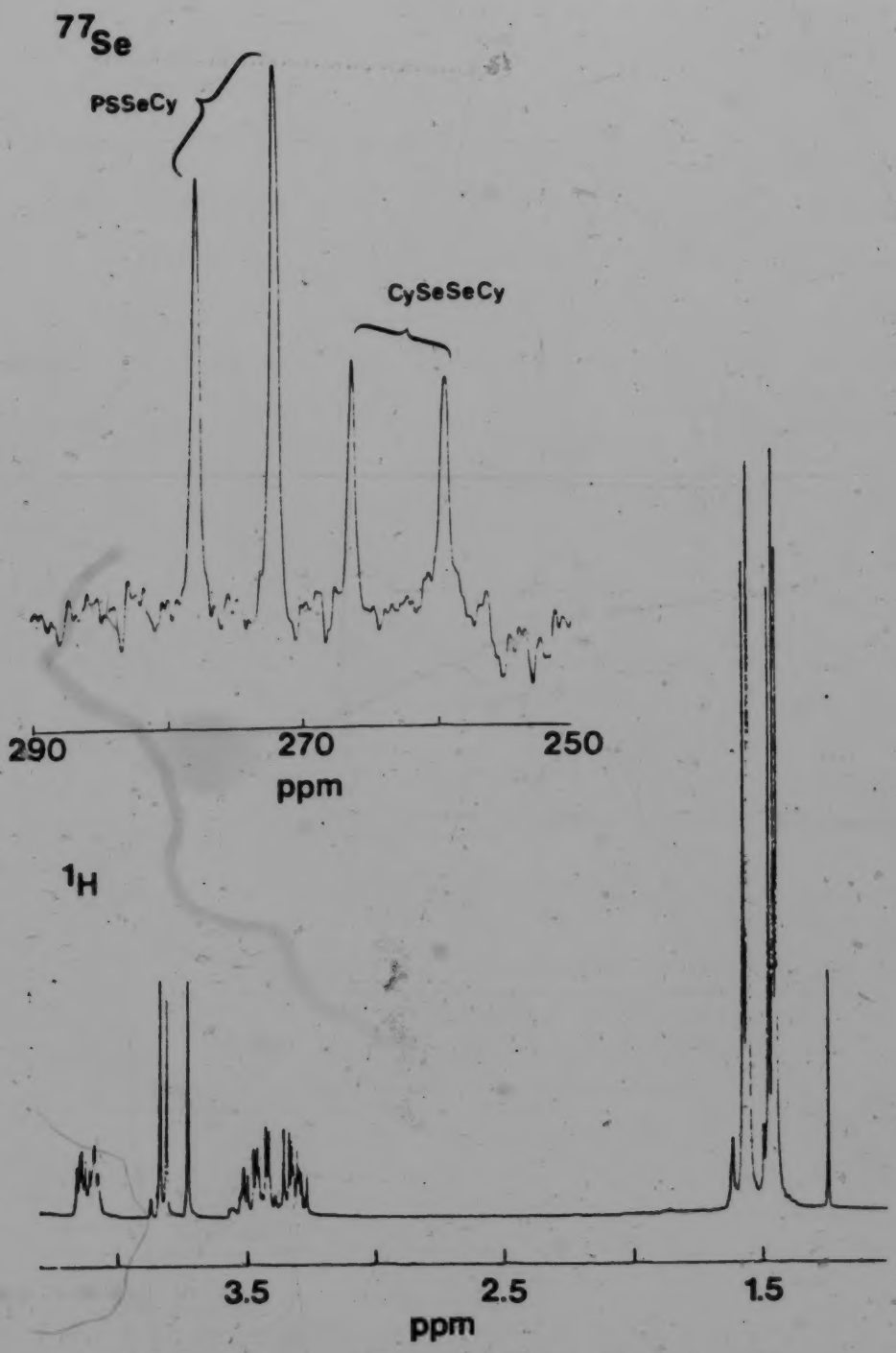


Figure 38. A: 360 MHz ^{1}H NMR and B: 68.68 MHz ^{77}Se NMR spectra of a pH* 3.68 solution obtained from the reaction of 0.088 M D-penicillamine with 0.090 M D-,L-selenocystine.

overlapping of the resonances of glutathione disulphide, GSSG, and the selenenyl sulphide, GSSeC. The ^{77}Se NMR spectrum shows only two resonances; the resonance which is at lower frequency was assigned to selenocystamine by comparison of the chemical shift to that of an authentic sample of selenocystamine at the same pH*. The resonance at higher frequency is assigned to the selenium in GSSeC. Figure 38 shows sharp resonances in both the ^{1}H and ^{77}Se NMR spectra obtained for a solution prepared by reaction 0.088 M D-penicillamine with 0.090 M D-,L-selenocystine at pH* ~9 followed by aeration and acidification to pH* 3.68. The ^{1}H NMR spectrum shows considerable overlap of the resonances of selenocystine and the mixed Se-S compound, although the methyl and methine resonances of the penicillamine portions are well resolved. The resonances observed in the ^{77}Se NMR spectrum could be assigned by comparing the chemical shifts that were observed with those obtained for selenocystine at this pH* (see Figure 30). The higher frequency pair of resonances was assigned to the selenenyl sulphide. As for the diselenide, separate resonances are observed for the stereoisomeric D-PSSeCy-D and D-PSSeCy-L species. The selenenyl sulphide and the diselenide are the only selenium-containing species for which ^{77}Se resonances are observed in these spectra. In all cases, the selenenyl

sulphide resonance is located to high frequency of the diselenide resonance. The resonances for the alkyl selenenyl sulphides are located fairly close to the resonances for the respective diselenides, i.e. between 200 to 400 ppm. The chemical shifts which have been determined for alkyl selenenyl sulphides in this work are listed in Table 17.

The ^{77}Se NMR spectra for solutions containing the following selenenyl sulphides are shown in Figure 39.

A: $^{-}OC_2CHCH_2SeSC(CH_3)_2CHCO_2^{-}$ (with $^{+}NH_3$ and $^{+}NH_3$)

B: $^{-}O_2CCHCH_2CH_2CN-CH-CNCH_2CO_2^{-}$ (with $^{+}NH_3$, H, CH_2, H, $SSeCH_2CHCO_2^{-}$, $^{+}NH_3$)

C: $^{-}O_2CCHCH_2SeSC(CH_3)_2CHCO_2^{-}$ (with $^{+}NH_3$, $HNCCH_3$, O)

In Spectra A and B resonances for stereoisomeric diselenide and selenenyl sulphide species could be clearly resolved, as indicated by two resonances for each species. In spectrum C for the third alkyl selenenyl sulphide, the isomeric species could not be resolved due

Table 17. ^{77}Se chemical shifts of alkyl selenenyl sulphides.

Alkyl Selenenyl Sulphide	Chemical Shift (ppm)	pH*
CSeSC	322.66	8.75
CSeSG	332.77	9.38
nPSSeSC	277.55	<5.0
CSeSP-D	281.97	7.80
$HOCH_2CH_2SeSP$-D	275.58	1.12
D-,L-CySeSCy-L	269.08 264.28	9.39
D-,L-CySeSG	333.10 326.66	3.65
D-,L-CySeSP-D	277.94 272.10	3.68
nPSSeCyS-D,-L	centered at ~263.50	9.38
D-,L-CySeSC	310.51	9.73

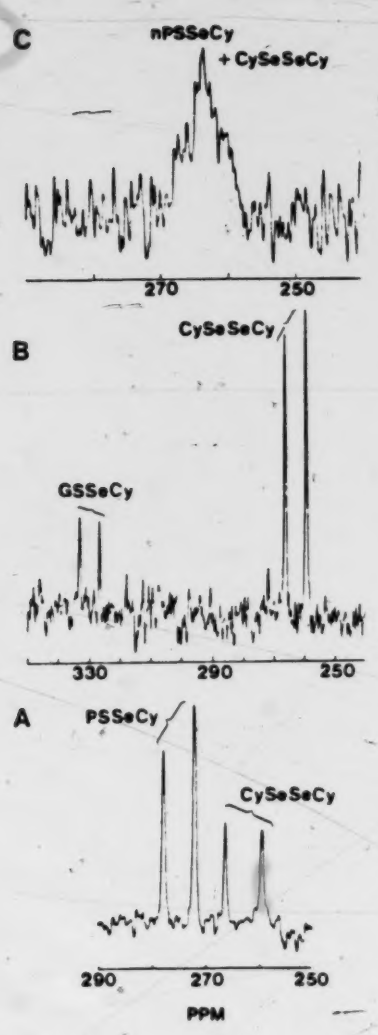


Figure 39. 68.68 MHz ^{77}Se NMR spectra of a mixture obtained by the reaction of

A: 0.088 M D-penicillamine with 0.090 M D-,L-selenocystine

B: 0.050 M glutathione with 0.045 M D-,L-selenocystine

C: 0.1159 M (D-,L-)N-acetylpenicillamine with 0.050 M D-,L-selenocystine.

to considerable overlap of the resonances. That stereoisomeric species can be detected in a mixture demonstrates the high sensitivity of the ^{77}Se chemical shift to very small variations in its chemical environment. Due to the instability of the selenenyl sulphides, their solution chemistry was not investigated by ^{77}Se NMR.

D. Discussion

The results which have been presented in this chapter show the usefulness of NMR spectroscopy for the study of the reaction of thiols with diselenides and selenols with disulphides. Two criteria for observing sharp resonances in NMR spectra of selenols have been established:

1. At pH* values above 2.5, the selenol solution must not contain any diselenides or selenenyl sulphides, because exchange reactions will broaden the NMR spectra.
2. ^{1}H NMR spectra showing sharp, separate resonances for both selenol and diselenide or selenol and selenenyl sulphide can be obtained by lowering the pH* to less than 2.5. Under these conditions ^{77}Se NMR spectra still exhibit very broad resonances.

The above criteria also apply when obtaining NMR spectra for diselenides and selenenyl sulphides. Solutions should be selenol-free, in this case.

To obtain ^{77}Se NMR spectra within a reasonable time, i.e. less than 90 minutes, solutions of selenium-containing compounds should have concentrations of at least 10 mM in selenium. Most reports of ^{77}Se NMR measurements have been made on samples enriched in the selenium isotope or on solutions with at least a tenth molar concentration in selenium.

NMR spectroscopy provides a technique for studying a complex mixture at the molecular level. Although considerable overlap of ^{1}H resonances are observed for compounds which are similar, there are systems in which the solution chemistry have been successfully studied; an example is provided by the elucidation of the acid-base chemistry of

$$^{-}O_2CCHC(CH_3)_2SSeCH_2CHCO_2^{-}$$
$$\quad\quad\quad

shift-pD data to the appropriate acid dissociation equilibria as detailed in Chapter 2.

^{77}Se NMR measurements made in this study have proven that this is a very sensitive technique for monitoring the selenium nucleus in selenium-containing compounds. In all cases studied, the ^{77}Se NMR spectra show relatively simple resonances compared to spectra obtained by ^{1}H NMR. Although the ^{77}Se chemical shift range is considerably large and finding the resonances for new species can be difficult, careful inference based on existing chemical shift data can decrease the time spent searching for the ^{77}Se resonances. This high sensitivity of chemical shift to the chemical structure is an advantage, however, because isomeric species present in solution together can be separately identified and studied simultaneously through their ^{77}Se resonances without the necessity of separating them, as is required in most other analytical techniques.

In characterizing the solution chemistry, in particular the acid-base chemistry, of the diselenides, the diselenodiethanoic acid system was closely investigated by both ^{1}H and ^{77}Se NMR techniques. By curve fitting chemical shift-pH* data sets, the acid dissociation constants for the diselenide could be obtained. The results show that ^{77}Se NMR can be used with

comparable accuracy for purposes of characterizing the acid-base chemistry of selenium-containing compounds with dissociable protons. In Chapter 3, the ^{77}Se chemical shift of the selenol, selenocysteamine, was measured as pH* was varied. The large chemical shift changes observed in the selenol deprotonation further indicates the high sensitivity of the ^{77}Se chemical shift to changes in chemical state.

The simplicity of ^{77}Se NMR spectra, as compared to the complexity of the ^{1}H NMR spectra, for many molecules can be used to advantage. For example, this has been demonstrated in this research by the determination of the acid dissociation constants for selenocystamine and the stereoisomers of selenocystine.

CHAPTER 5

THE REACTION OF SELENITE WITH THIOLS

A. Introduction

The reaction of selenite with biological thiols is thought to be a principal pathway for the incorporation of inorganic selenium into living systems. The reaction of selenious acid with thiols can be written as in equation 72. The reaction products are the disulphide, RSSR, and

$$4RSH + H_2SeO_3 \rightarrow RSSR + RSSeSR + 3H_2O \qquad [72]$$

the bis(alkylthio)selenide, RSSeSR. The thiols which have been studied include cysteine, glutathione, 2-mercapto-ethanol, co-enzyme A, and ribonuclease A (10-12). Of these, glutathione is particularly significant because it is one of the most abundant thiols in biological systems. For all the thiols studied, equimolar amounts of the disulphide and bis(alkylthio)selenide are formed, when selenium and thiol are reacted in the 1:4 stoichiometric amounts. Ganther found the rate of reaction of selenious acid with thiols over the pH range 1.2 to 6.4 to be most

rapid at low pH.

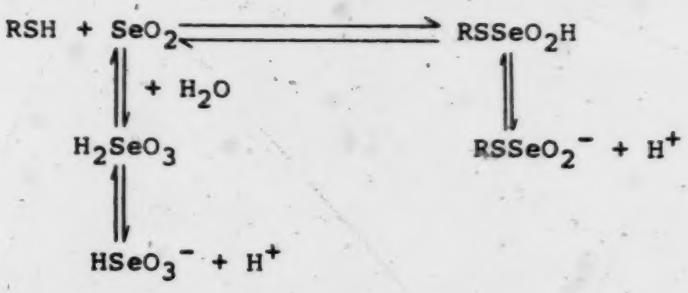
Results from Ganther's studies (12) on the reaction of selenious acid with glutathione show that the reaction is dependent upon pH and the selenium-thiol ratio. The pH dependency of the reaction of H_2SeO_3 with glutathione was studied by absorbance measurements at 260 nm, over the pH range 1.2 to 5.8. The results show that at pH 1.2, the reaction proceeds very rapidly and is complete in less than one minute. Between pH 1.2 and 4.7, absorbance measurements stabilized at a higher value compared to that at pH 1.2. Above pH 4.7, the initial absorbance is highly elevated, but decays to that obtained at pH 1.2. It was concluded from these observations that one or more intermediates are being formed.

Using stoichiometric amounts of selenium and thiol, the bis(alkylthio)selenide formed was found to be relatively stable in the pH range 1.2 to 5.8 studied. When the glutathione-selenium ratio exceeded 4:1, as would be the case in biological systems, it was found that the bis(alkylthio)selenide is unstable. Ganther proposed that further reaction occurs according to equation 73.

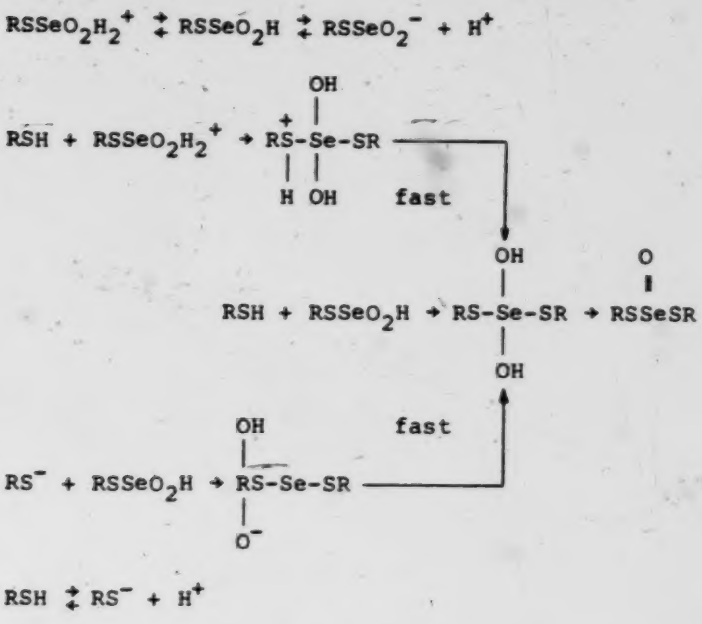
$$GSSeSG + GSH \rightarrow GSSeH + GSSG \qquad [73]$$

Although Ganther provides evidence for the formation of bis(alkylthio)selenides and some qualitative information about the rate of reaction of selenious acid with glutathione, there is little information available on the mechanism of reaction and the solution chemistry of the selenium-containing compound which is formed. More than a decade after the studies by Ganther, the reaction of selenite with thiols was again studied. Using conventional and stopped flow spectrophotometry, Kice examined the mechanistic pathway of the reaction (14,173). A model system consisting of the reaction of simple alkane thiols (n-butyl thiol and t-butyl thiol) with selenious acid in 60% aqueous dioxane was used. The rate of reaction was determined over the pH range from 0.2 to 8.1, with the thiol present in large excess over the selenious acid. The mechanism proposed for the reaction occurs in two stages and the reactions involved are given in Schemes I and II.

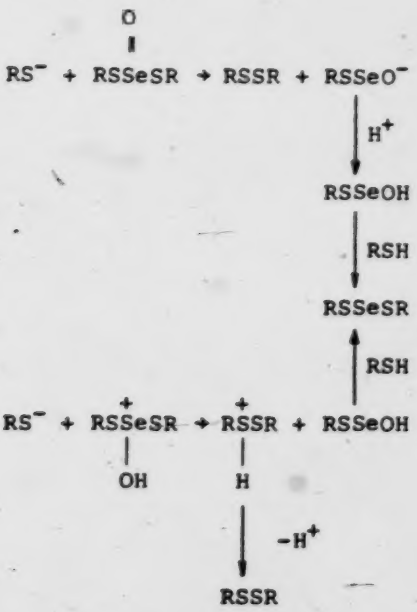
SCHEME I. Mechanism of the First Stage of the Reaction of Selenious Acid with Thiol (from Reference 14).



SCHEME IIa. Mechanism of the Second Stage of the Reaction of Selenite with Thiol (from Reference 14).


RSSeO2H2+ ⇌ RSSeO2H ⇌ RSSeO2− + H+
RSH + RSSeO2H2+ → RS-Se-SR (OH, H, OH)
fast
RSH + RSSeO2H → RS-Se-SR (OH) → RSSeSR (O)
RS− + RSSeO2H → RS-Se-SR (OH, O−)
fast
RSH ⇌ RS− + H+

SCHEME IIb. Mechanism for the Reaction of Thiol with RSSeSR (from Reference 174).



In the first stage of the reaction mechanism, Kice proposed that the reactive species is selenium dioxide, SeO_2, which is present in a small but significant amount in equilibrium with selenious acid. The intermediate species formed is the alkylthioseleninic acid, $RSSeO_2H$. Based on results obtained by Kice in earlier studies of the reaction of seleninic acid with thiols (174), the alkylthioseleninic acid thus formed in the first stage of the reaction of selenious acid with thiols is expected to be converted to a seleninyl sulphide, RSSeSR, as depicted in Scheme IIa. This species is then converted to the bis-(alkylthio)selenide by further reaction with another thiol molecule, according to the reactions in Scheme IIb.

Kice also suggests that other reasonable pathways for the formation of the bis(alkylthio)selenide from RSSeSR include the following reactions:

$$RSH + RSSeSR \rightarrow RSOH + RSSeSR \quad [74]$$

$$RSSeSR \xrightarrow{\text{isomerization}} RSSeOSR \xrightarrow{RSH} RSOH + RSSeSR \quad [75]$$

It was observed in the above study that the reaction

of selenite with a tertiary thiol also yields a thiol sulphonate, $RSSO_2SR$ besides the disulphide and the bis-(alkylthio)selenide. It was suggested that primary and tertiary thiols favour different reaction pathways dependent upon their structural differences.

An interesting observation was made in a study of the selenenate (RSeOH)-selenoxide (RSeH) equilibrium (175). It was found that the selenenate form is the thermodynamically more favoured, compared to the selenoxide; it is therefore proposed that in the equilibria between the RSSeSR and RSSeOSR species, the latter would be the favoured species, i.e.

$$\text{RSSeSR} \rightleftharpoons \text{RSSeOSR} \qquad [76]$$

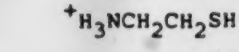
This suggests that the reaction mechanism proposed by Kice as given in Scheme IIb may not be correct for the reaction of selenite with thiols.

That bis(alkylthio)selenides can be formed by the reaction of selenite with thiol groups in biologically important molecules suggests this as an important pathway for the initial incorporation of selenium into living systems; in the presence of two or more different thiol

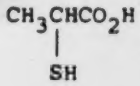
groups, then asymmetrical molecules, RSSeSR' could be formed. This reaction has been further investigated in this work by NMR spectroscopy. As has been indicated in the earlier chapters, NMR spectroscopy offers the advantages of providing information at the molecular level and ease of characterizing the solution chemistry of a compound without having to obtain a pure sample of it. In this study, the latter advantage is particularly important in view of the instability of the bis(alkylthio)selenide species. Both ^{1}H and ^{77}Se NMR spectra were obtained of the products of the reaction of selenite with thiols. The names and structures of the thiols which have been used in this study are given in Figure 40.

The feasibility of studying the reaction of selenite with thiols was first examined by ^{1}H NMR spectroscopy. ^{1}H NMR spectra were measured for mixtures obtained by reacting cysteamine, 2-mercaptopropanoic acid, D-penicillamine and glutathione with selenite and the resonances for the bis(alkylthio)selenides were assigned. The solution chemistry, in particular, the acid-base chemistry, of the bis(alkylthio)selenides formed by the reaction of the first three thiols with selenite were characterized; the ^{1}H NMR spectra of reduced and oxidized glutathione and bis(glutathione)selenide were too complex due to ^{1}H spin-spin coupling, hence, the acid-base

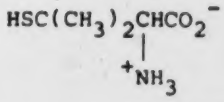
1. Cysteamine (CSH)



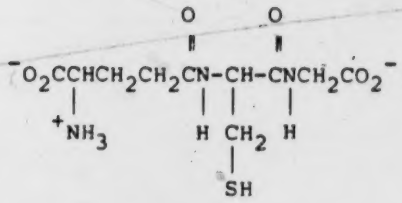
2. 2-Mercaptopropanoic Acid (2-MPA)



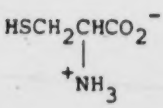
3. Penicillamine (PSH)



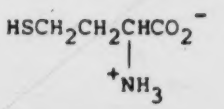
4. Glutathione (GSH)



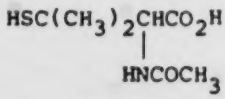
5. Cysteine (CySH)



6. Homocysteine (hCySH)



7. N-Acetyl Penicillamine (nPSH)



8. Mercaptosuccinic Acid

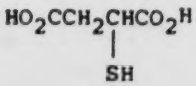


Figure 40. Structures and names of thiols used in this study.

chemistry of bis(glutathione)selenide was not examined by ^1H NMR. In most cases, the ^1H NMR spectra; for example, for asymmetrical molecules, RSSeSR', are too complicated due to overlapping resonances to allow characterization of the acid-base chemistry by ^1H NMR spectroscopy.

Since no ^{77}Se NMR spectra of bis(alkylthio)selenides have been reported, the ^{77}Se resonances were first located and their chemical shifts obtained. ^{77}Se NMR spectra for symmetrical, RSSeSR, and asymmetrical, RSSeSR', molecules were obtained. The use of ^{77}Se NMR as a probe to characterize the acid-base chemistry of the bis(alkylthio)selenide was investigated.

B. Results From ^1H NMR Measurements

1. Reaction of Cysteamine with Selenite

The reaction of cysteamine with selenite was studied at 25°C in D_2O using concentrations of thiol to selenite in the 4:1 ratio. This reaction was studied over the pH* range from 0.7 to 7.0. Figure 41 shows a pH* 4.6 ^1H NMR spectrum of a reaction mixture (Spectrum B in Figure 41) obtained by the reaction of cysteamine with selenite in a 4:1 ratio. Two pairs of triplet resonances for the reaction products are observed. At pH* values below 4.6, spectra measured at 2 minutes after the start of the

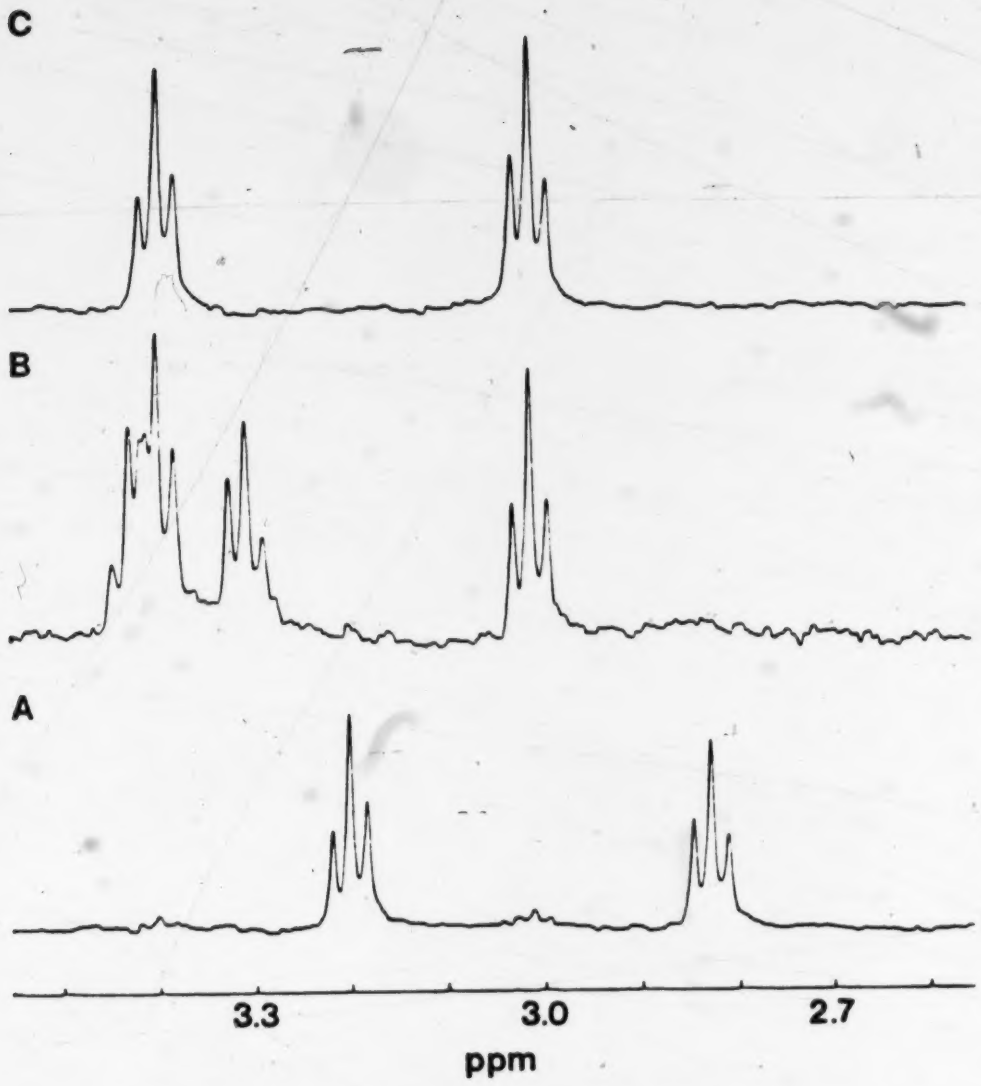


Figure 41. The methylene region of 360 MHz ^{1}H NMR spectra of
A: 0.19_8 M cysteamine
B: a solution prepared by reacting 0.16_5 M cysteamine with 0.04_2 M selenite
C: 0.09_9 M cystamine
(The pH* of all solutions was 4.6.)

reaction show two pairs of resonances. As supporting evidence, a ^{13}C NMR spectrum of a reaction mixture at pH* 2.8 (Spectrum B in Figure 42) also show two pairs of resonances from the products of the reaction. A comparison of the ^{1}H and ^{13}C spectra with those of authentic samples of cysteamine (Spectrum A in Figures 41 and 42) and cystamine (Spectrum C in Figures 41 and 42) under the same reaction conditions show that no cysteamine is present in the reaction mixture; and one of the products is cystamine. The reaction proceeds very rapidly throughout the pH* range studied, as no thiol resonances are observed at pH* below 4.8 and above pH* 5.2. To illustrate this, ^{1}H NMR spectra obtained at various times after the start of the reaction of 13.32 mM cysteamine with 3.33 mM selenite in D_2O at pH* 2.5, are shown in Figure 43. These spectra show that after 5 minutes, the resonances were invariant with respect to chemical shift and intensity. At pH* 4.8, however, resonances due to small quantities of cysteamine were also detected at 2 minutes after the reaction had been initiated; at pH* 5.2, these resonances were not detectable. From relative intensity estimations, the overall rate of reaction appears to decrease to a minimum at pH ~4.8, but increases again at pH* higher than 5.2. A large amount of red elemental selenium was obtained at alkaline pH*; ^{1}H NMR

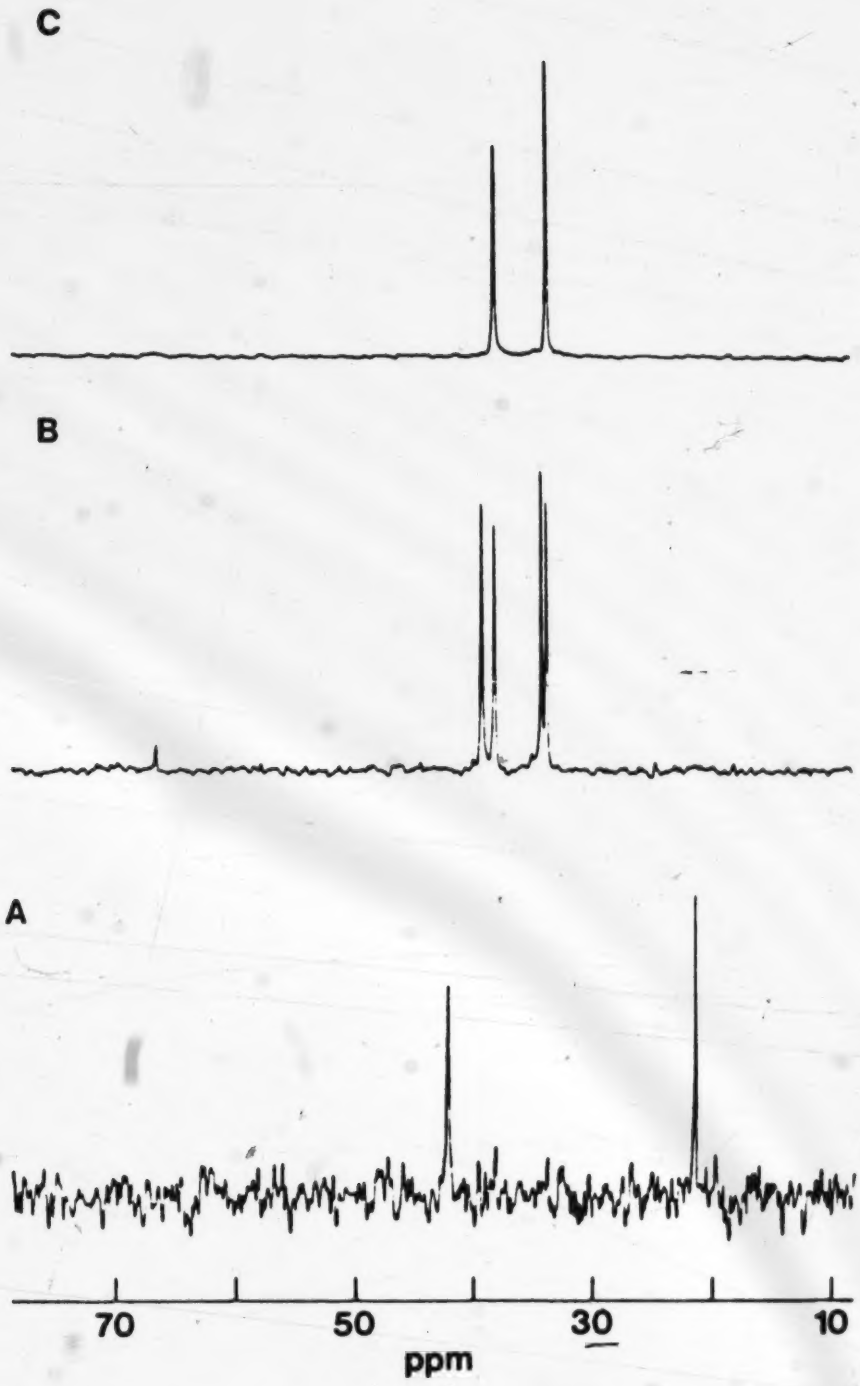


Figure 42. 75.47 MHz ^{13}C NMR spectra of A: 0.19_8 M cysteamine, B: a solution prepared by reacting 0.16_5 M cysteamine with 0.04_2 M selenite, and C: 0.09_9 M cystamine. (The pH* of all solutions was 2.8.)

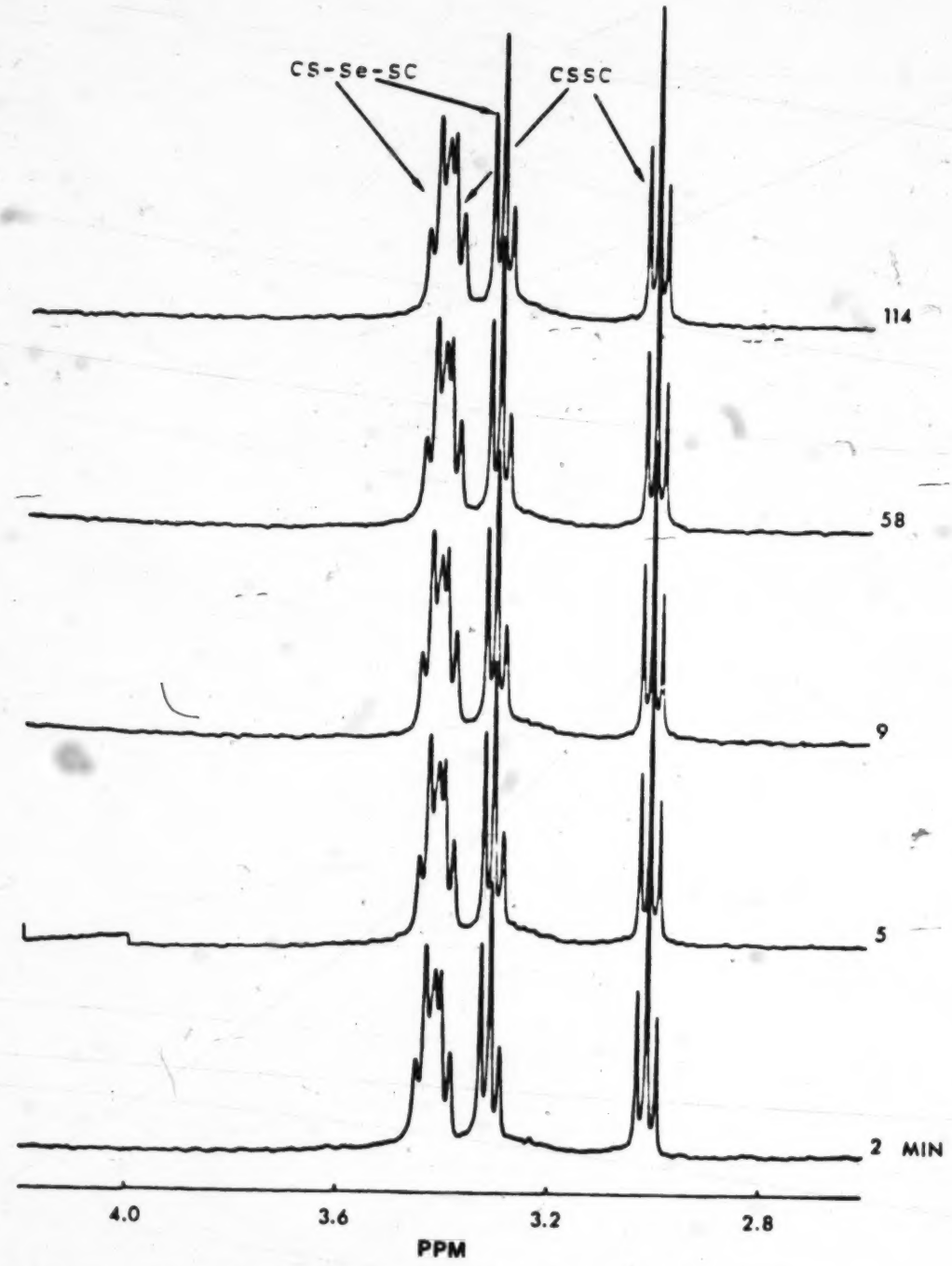


Figure 43. The methylene region of the 360 MHz ^{1}H NMR spectra obtained as a function of time for the reaction of 13.32 mM cysteamine with 3.33 mM selenite at pH* 2.5.

spectra obtained show only resonances for cystamine in all cases, which is in accord with the decomposition of bis(cysteamine)selenide under alkaline conditions.

In order to characterize the acid-base chemistry of bis(cysteamine)selenide by ^1H NMR spectroscopy, a D_2O solution of 9.86 mM cysteamine was reacted with a D_2O solution of 2.50 mM selenite; the chemical shift of the methylene protons closer to the selenium atom in bis(cysteamine)selenide, CSSeSC, was monitored as pH* was varied. The system was left to equilibrate at each pH* and chemical shift-pH* data for CSSeSC were then obtained. Table 18 lists the chemical shifts of the central peaks of the two methylene resonances of bis(cysteamine)selenide and of cystamine obtained from this titration. The chemical shift of one of the methylene proton resonances of bis(cysteamine)selenide is plotted as a function of pH* in Figure 44. The acid dissociation equilibria and constants for bis(cysteamine)-selenide are represented as

$$H_2A^{2+} + H_2O \rightleftarrows H_3O^+ + HA^+$$

$$K^D_1 = \frac{[H_3O^+][HA^+]}{[H_2A^{2+}]}$$

$$HA^+ + H_2O \rightleftarrows H_3O^+ + A$$

Table 18. Assignment and ^1H chemical shift data of methylene proton resonances in Figure 43.

pH*	CSSeSC		CSSC	
5.53	3.4302	3.3109	3.4024	3.0140
6.00	3.4302	3.3109	3.4024	3.0140
6.85	3.4289	3.3102	3.4011	3.0133
7.08	3.4275	3.3089	3.3997	3.0126
7.31	3.4255	3.3089	3.3984	3.0120
8.17	3.3950	3.2940	3.3726	2.9998
8.32	3.3821	3.2885	3.3638	2.9964
8.46	3.3706	3.2824	3.3523	2.9909
8.57	3.3577	3.2790	3.3401	2.9848
8.99	3.2872	3.2580	3.2757	2.9570
9.19	3.2438	3.2357	3.2357	2.9381
9.68	3.1638	3.1347	3.0170	2.8845
9.83	3.1489	3.1067	3.6825	2.8695
10.02	3.1306	3.0709	3.0418	2.8520
10.24	3.1164	3.0384	3.0038	2.8357
10.56	3.1021	3.0177	2.9645	2.8194
10.74	3.0974	2.9970	2.9516	2.8140
10.91	3.0913	2.9869	2.9387	2.8079
11.24	3.0879	2.9781	2.9259	2.8032
11.74	3.0845	2.9706	2.9157	2.7984

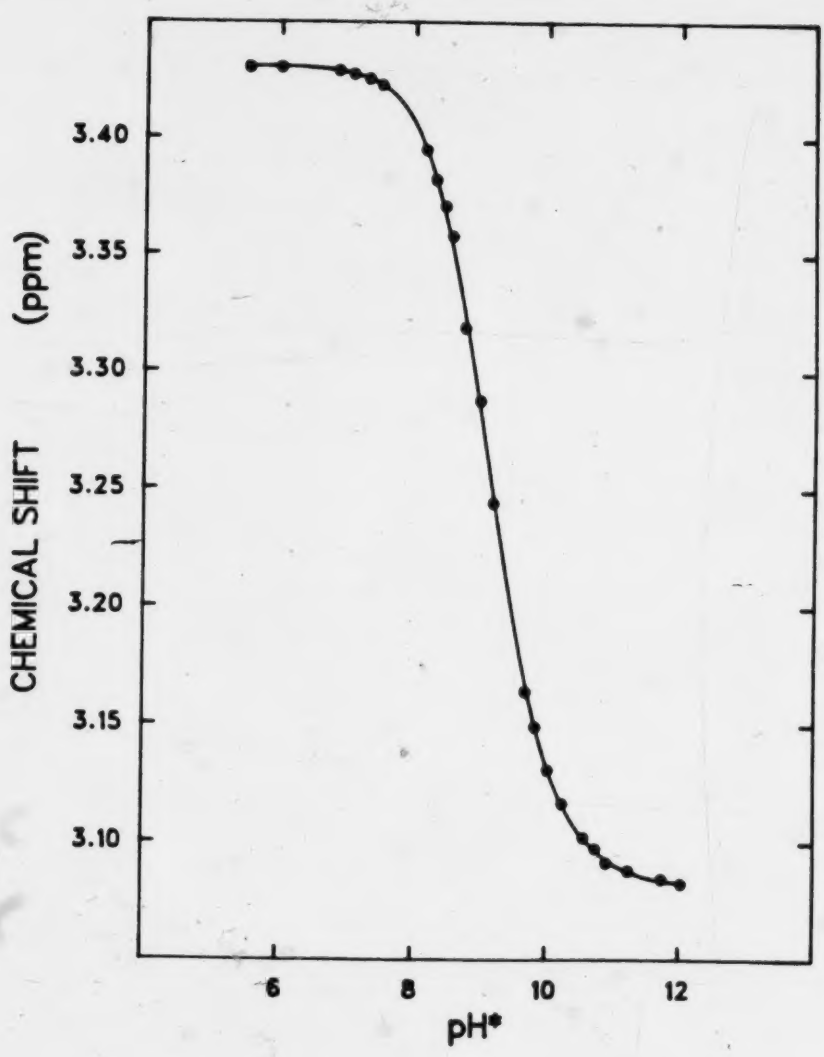


Figure 44. Variation of the chemical shift of the resonance for the methylene protons adjacent to the selenium of bis(cysteamine)selenide with pH*.

$$K^{D}_{2} = \frac{[H_3O^+][A]}{[HA^+]}$$

Using the non-linear least squares curve fitting routine KINET, the acid dissociation constants for bis-(cysteamine)selenide, K^{D}_{1} and K^{D}_{2}, were calculated from the chemical shift data. The amino pK^{D}_{A}'s were estimated to be

$$pK^{D}_{1} = 9.08 \pm 0.01$$

$$pK^{D}_{2} = 10.66 \pm 0.04$$

Due to the instability of bis(cysteamine)selenide at pH* values above 8.0, as well as errors due to overlapping methylene resonances, the uncertainty in the measurement of the chemical shift was larger at higher pH* readings. The chemical shifts for fully protonated and deprotonated bis(cysteamine)selenide are 3.4309 ± 0.0009 ppm and 3.0818 ± 0.0019 ppm, respectively.

2. Reaction of 2-Mercaptopropanoic Acid with Selenite

D_2O solutions of 2-mercaptopropanoic acid and selenite were mixed in the 4:1 ratio over the pH* range 1.8 to 7.9. The reaction of selenite with this secondary

thiol proceeds quickly to an equilibrium situation, in which thiol, disulphide, and bis(alkylthio)selenide are all present as indicated by resonances for thiol (RSH), disulphide (RSSR), and bis(alkylthio)selenide (RSSeSR). Figure 45 shows the entire spectrum obtained for a pH* 3.02 solution prepared by the reaction of 2-mercaptopropanoic acid with selenite. The methine proton region shows overlapping resonances at ~3.8 ppm due to stereoisomeric species of RSSeSR. The inset is an expanded plot of the methyl proton region which is used in this study for the characterization of the RSSeSR molecule. The methyl resonance is a doublet due to spin-spin splitting by the methine proton. The assignment of the resonances was by comparison of the spectra obtained for the reaction mixture with the spectra of authentic samples of the thiol and the disulphide under the same reaction conditions. The highest frequency doublet was assigned to RSSeSR. The linewidths of the RSSeSR doublet are larger, in comparison to those of the other doublets. This is most likely due to the overlap of the resonances for the D-,D-(L-,L-) and D-,L-(L-,D-) stereoisomers of this molecule. Although the methyl doublet resonances for the RSH, RSSR, and RSSeSR species overlap, they are resolved sufficiently for peak assignment; resonances for the two stereoisomers of the

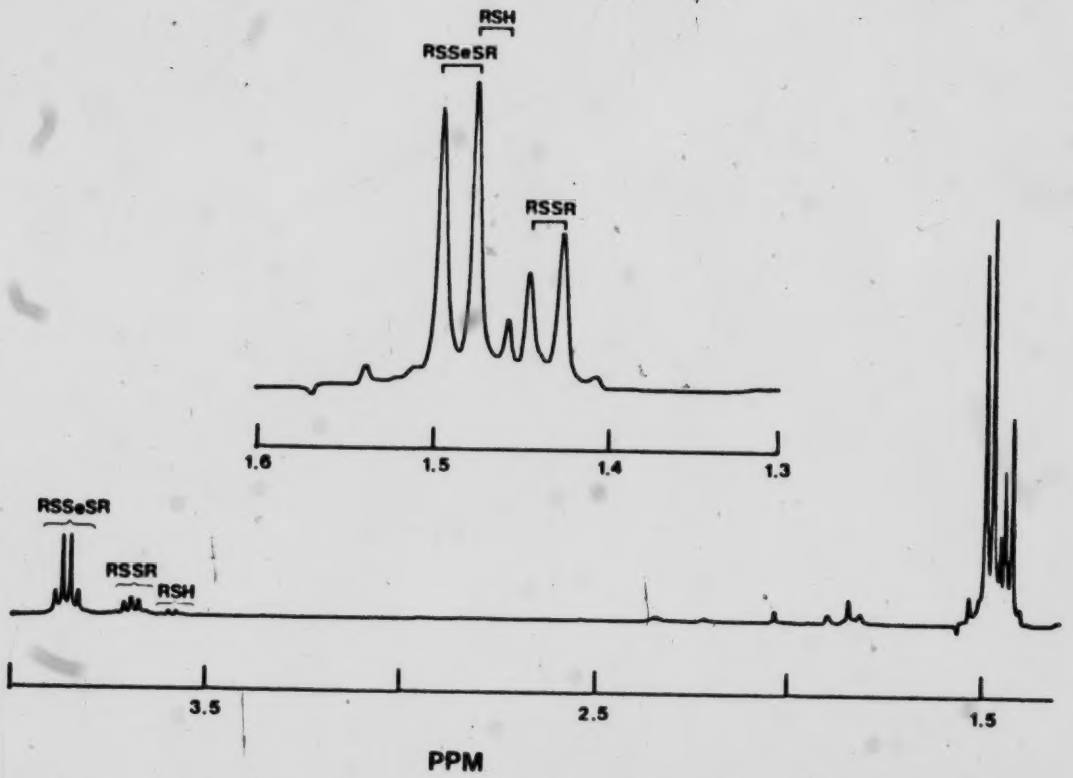


Figure 45. 360 MHz ^{1}H NMR spectrum of a mixture prepared by the reaction of 10.23 mM 2-mercaptopropanoic acid with 2.51 mM selenite at pH* 3.02. The inset is an expanded region of the methyl proton region of the spectrum.

disulphide are observed. Throughout the entire pH* range, slight decomposition of the selenium-containing product occurs, as evidenced by the precipitation of red elemental selenium.

In order to determine if quantitative results can be extracted from relative intensity measurements in the above experiments, spin lattice relaxation times were estimated for the methyl resonances of RSSeSR and RSSR at pH* 4.78. The average T_1 value for the methyl protons of RSSeSR is 0.75 seconds and 1.05 seconds for those of RSSR. Spectra were obtained under conditions where the repetition time for acquiring a spectrum were too short to allow complete relaxation of the magnetization so that quantitative results cannot be obtained from relative intensity measurements. Hence, only the qualitative conclusion that the reaction of 2-mercaptopropanoic acid with selenite appears to be slower than the reaction of cysteamine with selenite, since resonances for the thiol can be detected at all acidic pH* values, can be made. Figure 46 shows ^1H NMR spectra measured as a function of time after the start of reaction of 10.23 mM 2-mercapto-propanoic acid and 2.51 mM selenite at pH* 5.30. The spectral region plotted in Figure 46 shows only the methyl resonances for the thiol, disulphide and bis(alkylthio)-selenide.

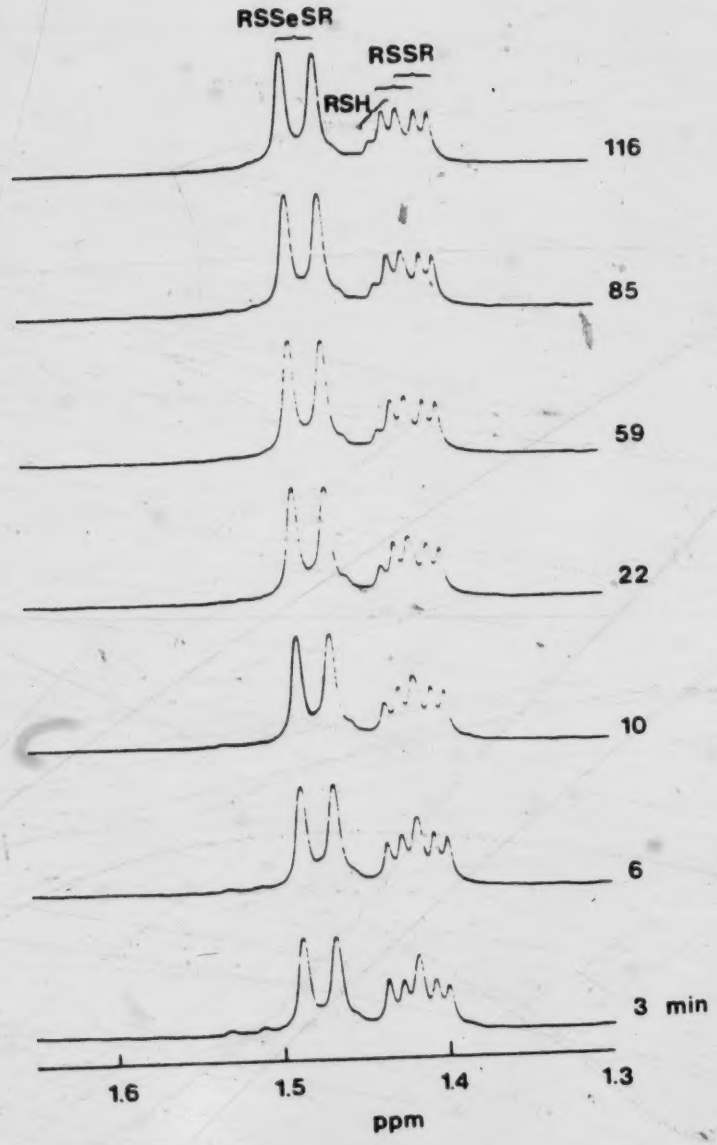


Figure 46. The methyl region of 360 MHz ^{1}H NMR spectra obtained as a function of time for the reaction of 10.23 mM 2-mercapto-propanoic acid with 2.51 mM selenite at pH* 5.30.

Since the methyl resonances for RSSeSR can be resolved, the acid-base characteristics of bis(2mercapto-propanoic acid)selenide were studied by observing the variation of the chemical shift of the methyl resonances of this compound with pH*. A reaction mixture containing initial thiol and selenite concentrations of 12.12 mM and 3.03 mM, respectively, was allowed to react for 30 minutes until equilibrium was reached, before titration was begun. Table 19 lists the ^1H chemical shifts of each doublet component of the RSSeSR, RSSR, and RSH resonances observed in this titration. From the chemical shift-pH* data obtained, the carboxylic acid dissociation constants of the RSSeSR species were determined by non-linear least squares curve-fitting to be

$$pK^D_1 = 3.17 \pm 0.02$$

$$pK^D_2 = 4.48 \pm 0.06$$

3. Reaction of D-Penicillamine with Selenite

The reaction of high concentrations of D-penicillamine and selenite (0.2 M D-penicillamine and 50 mM selenite), at pH* 6.0 yielded a precipitate which, after filtration and recrystallization from water, gives a ^1H NMR spectrum having resonances at 1.4914 ppm, 1.6148

Table 19. Assignment and ^1H chemical shift data of methyl proton resonances in Figure 46.

pH	RSSeSR		RSSR[a]				RSH	
			(1)		(2)			
2.31	1.5022	1.4827	1.4555	1.4358	1.4555	1.4358	1.4894	1.4697
2.53	1.5009	1.4819	1.4555	1.4358	1.4555	1.4358	1.4873	1.4677
2.70	1.5009	1.4812	1.4541	1.4345	1.4541	1.4345	1.4860	1.4663
2.90	1.4995	1.4805	1.4507	1.4317	1.4507	1.4317	1.4819	1.4629
3.02	1.4989	1.4799	1.4494	1.4304	1.4494	1.4304	1.4799	1.4609
3.20	1.4982	1.4772	1.4460	1.4270	1.4460	1.4270	1.4772	1.4568
3.40	1.4961	1.4772	1.4453	1.4243	1.4412	1.4243	1.4711	1.4514
3.61	1.4955	1.4765	1.4446	1.4216	1.4372	1.4175	1.4643	1.4446
3.80	1.4941	1.4744	1.4385	1.4189	1.4331	1.4134	1.4582	1.4385
4.01	1.4934	1.4738	1.4358	1.4161	1.4324	1.4100	1.4521	1.4324
4.22	1.4928	1.4704	1.4331	1.4141	1.4277	1.4073	1.4473	1.4277
4.40	1.4921	1.4724	1.4317	1.4128	1.4249	1.4053	1.4439	1.4249
4.61	1.4914	1.4717	1.4304	1.4114	1.4222	1.4031	1.4412	1.4222
4.84	1.4907	1.4711	1.4290	1.4094	1.4195	1.4019	1.4385	1.4195
5.30	1.4907	1.4711	1.4290	1.4094	1.4182	1.4012	1.4378	1.4182
6.32	1.4887	1.4697	1.4209	1.4080	1.4168	1.4006	1.4358	1.4168

[a](1) and (2) are the doublet resonances for the D-,D-(L-,L-) and the D-,L-(L-,D-) stereoisomers of the disulphide molecule.

ppm, and 3.3850 ppm (pH* 6.00). These resonances were assigned to the two methyl groups and the methine proton, respectively, of bis(D-penicillamine)selenide, D-PSSeSP-D. Elemental analysis indicates the bis(D-penicillamine)selenide monohydrate was obtained. (Calculated: C, 30.77%; H, 5.54%; O, 20.28%; N, 7.05%. Found: C, 30.53%; H, 5.64%; O, 20.34%; N, 7.12%.) It was observed that once bis(D-penicillamine)selenide was formed, it was more stable than bis(cysteamine)selenide and bis(2-mercaptopropanoic acid)selenide. No decomposition to red elemental selenium was observed after 60 days for a reaction mixture buffered at pH* 7.4 and the ^{1}H NMR spectrum shows the presence of D-penicillamine disulphide and bis(D-penicillamine)selenide. However, when a solution of this compound was heated to boiling, red elemental selenium was produced, indicating its instability at high temperatures.

Spin lattice relaxation times for the methyl resonances of D-penicillamine, D-,D-penicillamine disulphide and bis(D-penicillamine)selenide were determined at pH* 3.80 and are listed below:

Molecule	T_1(s) $CH_{3,1}$	$CH_{3,2}$
D-penicillamine	0.799 ± 0.004	0.841 ± 0.014
D-,D-penicillamine disulphide	0.328 ± 0.006	0.459 ± 0.010
Bis(D-penicillamine)selenide	0.320 ± 0.002	0.448 ± 0.002

For a repetition time of 1.024 s, the optimum flip angle used to obtain a spectrum was determined from equation 77 (176):

$$\cos \theta = \exp(-t_r/T_1) \qquad [77]$$

where θ is the flip angle, t_r is the repetition rate, and T_1 is the spin lattice relaxation time of the methyl resonance of D-penicillamine.

To investigate the stoichiometry of the reaction of D-penicillamine with selenite, the mole ratio of thiol to selenite was varied from 4:1 to 1:1 in an acetate buffered medium of pH* 4.26 and ^1H NMR spectra obtained. The spectral portions showing the methyl resonances at 5 minutes after the start of the reaction for various ratios of thiol to selenite are shown in Figure 47. In each case, it is noted that the amount of bis(D-penicillamine)-selenide formed is greater than the amount of disulphide

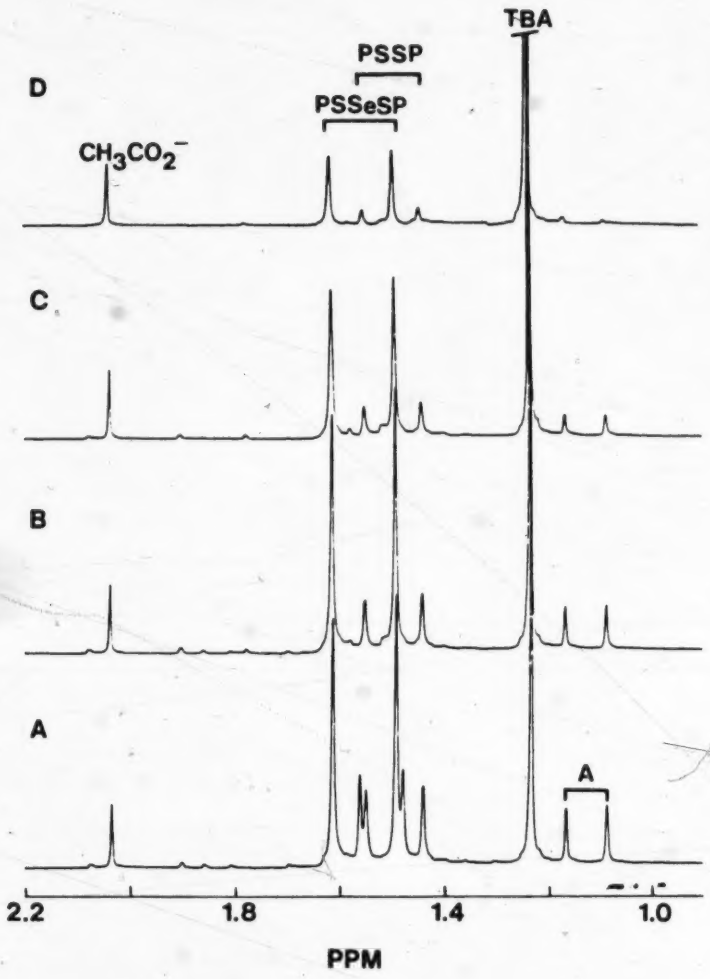


Figure 47. The methyl region of 360 MHz ^{1}H NMR spectra obtained from a mole ratio study of the reaction of D-penicillamine with selenite at various mole ratios in an acetate buffer. The ratio of D-penicillamine to selenite was A: 4:1, B: 3:1, C: 2:1, D: 1:1.

formed in the same time interval. Since the T_1 values for the disulphide and the bis(alkylthio)selenide are similar, the relative intensities can be directly related to the concentrations of these species. From relative intensity measurements, it is suggested that the reaction pathway proposed by Kice (14), according to which equimolar amounts of the disulphide and bis(alkylthio)selenide would be produced, may not completely describe the reaction of D-penicillamine with selenite.

The reaction of D-penicillamine with selenite was further examined. ^{1}H NMR spectra were obtained as a function of time for reaction mixtures having the stoichiometric 4:1 ratio of thiol to selenite, over the pH* range 1.4 to 7.0. An equilibrium mixture appears to be rapidly established under acidic conditions. To illustrate this observation, Figure 48 shows the methyl region of ^{1}H NMR spectra obtained from a time course study when 9.69 mM D-penicillamine was reacted with 2.50 mM selenite at pH* 2.42. Two methyl resonances each, due to the presence of the asymmetric C_α in these molecules, are observed for the thiol, disulphide and the bis(alkylthio)-selenide. From the relative intensities of the different methyl resonances, the concentration of thiol decreases with time while that of the disulphide increases, and that of bis(D-penicillamine)selenide increases slightly. Other

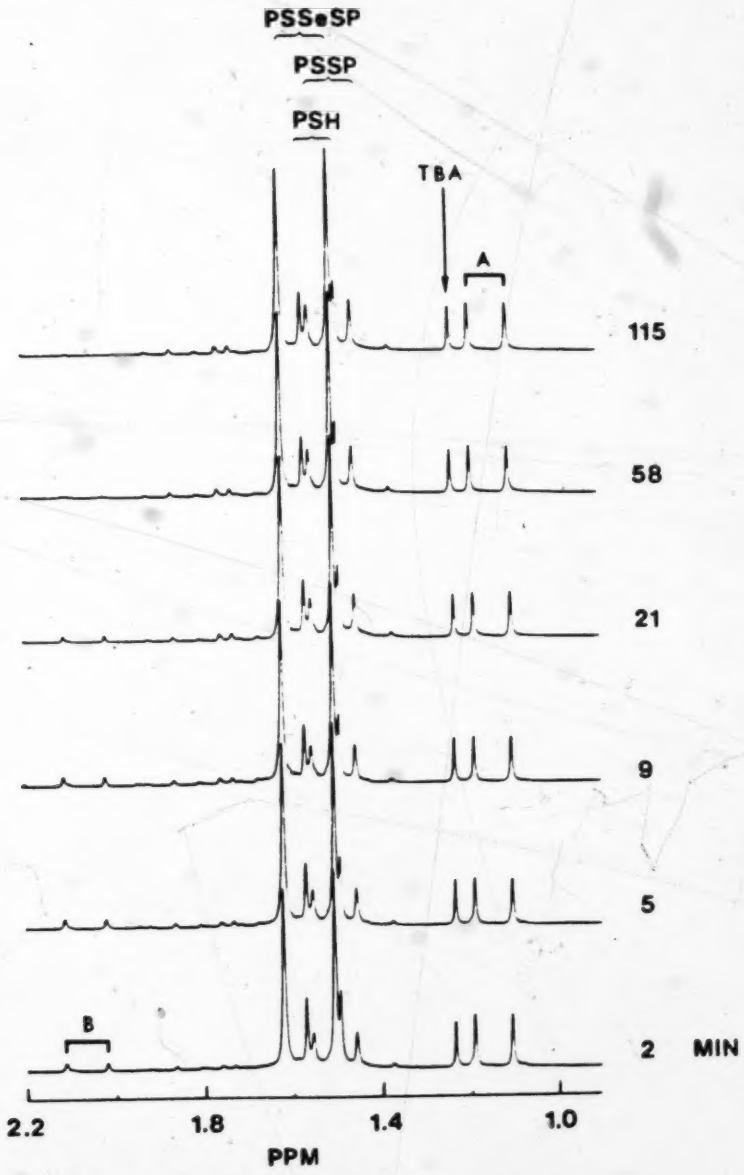


Figure 48. The methyl region of 360 MHz ^{1}H NMR spectra obtained as a function of time from a study of the reaction of 9.69 mM D-penicillamine with 2.50 mM selenite at pH* 2.42.

resonances, apparently for methyl groups, besides those which can be assigned to the above three molecules are observed at lower and higher frequencies (A and B). The intensities of the low frequency methyl resonances (between 1.0 ppm and 1.2 ppm), (A), do not change with time, although the higher frequency resonances (between 2.0 ppm and 2.2 ppm), (B), decreases during the first 60 minutes of the reaction. These high frequency resonances may be due to intermediates formed during the course of the reaction (see Schemes I and IIa).

To qualitatively compare the rates of reaction of D-penicillamine with selenite, ^{1}H NMR spectra obtained between 5 and 6 minutes after the start of the reaction are shown in Figure 49 over the pD range studied. As the relative intensities of the methyl resonances in the ^{1}H NMR spectra can be related to the concentrations of the species present in the reaction mixture, qualitative observation on the rate of reaction of D-penicillamine with selenite can be made. By comparing the relative intensities of the methyl resonances for D-penicillamine, the rate of reaction appears to decrease to a minimum at pD ~4.2, where the relative intensity of the methyl resonance of D-penicillamine is highest. The rate appears to increase again and at pD ~6.8, the methyl resonances of D-penicillamine are no longer observed.

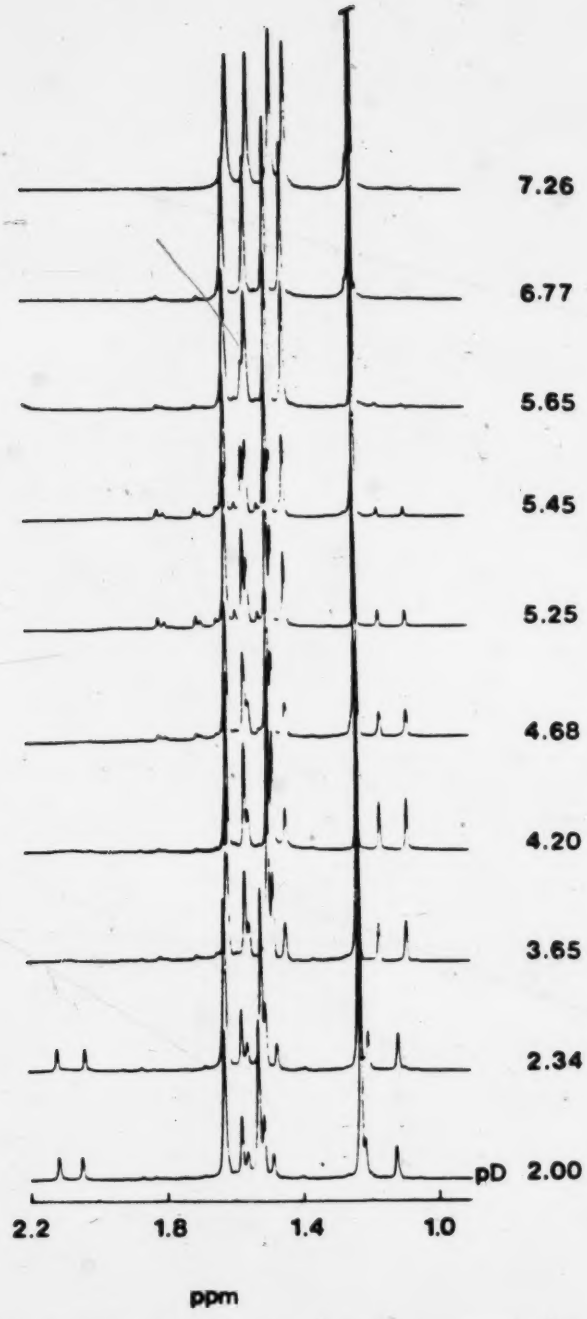


Figure 49. The methyl region of 360 MHz ^{1}H NMR spectra obtained 5-6 minutes after the start of the reaction of D-penicillamine with selenite (D-PSH:SeO_3^{2-} ≅ 10 mM:2.5 mM) at various pD values.

These spectra also show that at low pD values, additional methyl resonances are observed to higher and lower frequencies of those of the thiol, disulphide, and bis(alkylthio)selenide. As indicated in the preceeding paragraph, the high frequency resonances, (B), are probably due to reaction intermediates, since their intensities decrease with time. The low frequency resonances have yet to be assigned to any compound, although the variation of the chemical shifts of the methine resonances with pD show they possess acid-base characteristics similar to those of D-,D-penicillamine disulphide (see Figure 50). These lower frequency resonances have very low intensities above pD 5.4 and are absent above pD 6.4, and may be due to a product of a competing reaction occurring at more acidic pD values.

In order that the acid-base chemistry of bis(D-penicillamine)selenide may be characterized, a reaction mixture was prepared at pH* 1.4 and allowed to equilibrate for 30 minutes before it was titrated with KOD. During the course of the titration, the intensities of resonances thought to be due to reaction intermediates (B), as explained previously, gradually decrease while the intensity of those thought to be due to other by-products (A), remained stable from pH* 1.4 to 12.6. The resonances assigned to D-PSSeSP-D were well-defined over the entire

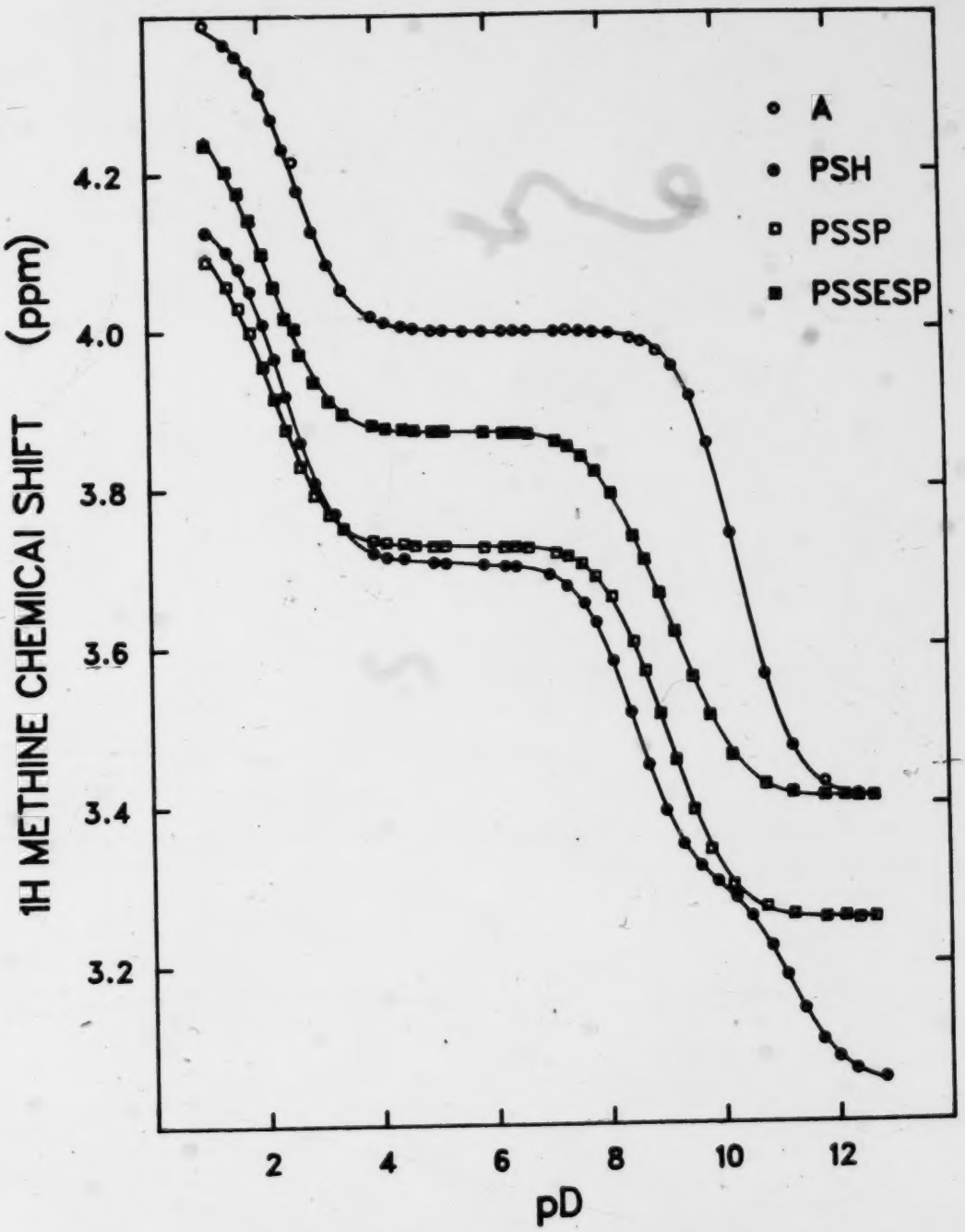


Figure 50. The chemical shifts of the resonances for the methine protons of D-penicillamine, D-,D-penicillamine disulphide, bis(D-penicillamine)selenide and the unidentified compound, A.

pH* range and the duration of the titration. Figure 50 is a plot of the ^1H NMR chemical shifts of the meth-ine protons of (A), D-penicillamine, D-,D-penicillamine disulphide, and bis(D-penicillamine)selenide. The acid dissociation constants for the ammonium groups of bis(D-penicillamine)selenide and the chemical shifts of the ammonium-protonated and deprotonated forms were estimated from the chemical shift-pD data of the methyl and methine resonances by non-linear least squares curve fitting. The following pK^D_A values and ^1H NMR limiting chemical shifts were obtained from the chemical shift data for the two methyl groups:

i. $pK^D_3 = 8.24 \pm 0.04$
$pK^D_4 = 9.56 \pm 0.02$
$\delta_{H_2L} = 1.6154 \pm 0.0003$
$\delta_L = 1.4642 \pm 0.0003$

ii. $pK^D_3 = 8.32 \pm 0.02$
$pK^D_4 = 9.48 \pm 0.05$
$\delta_{H_2L} = 1.4945 \pm 0.0002$
$\delta_L = 1.3481 \pm 0.0003$

The pK^D_A values estimated from methine chemical shift-pD data are:

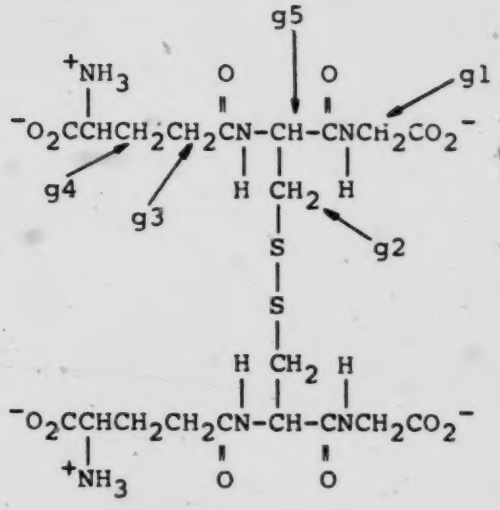
$pK^D_3 = 8.31 \pm 0.01$ $\quad$ $pK^D_4 = 9.55 \pm 0.01$
$\delta_{H_2L} = 3.8716 \pm 0.0003$ $\quad$ $\delta_L = 3.4087 \pm 0.0003$

From these results, the average values for the acid

dissociation constants of the ammonium groups of bis(D-penicillamine)selenide are 8.29 ± 0.04 and 9.53 ± 0.04.

4. Reaction of Glutathione with Selenite

Figure 51 shows the entire ^1H NMR spectrum measured for a pH* 4.33 D_2O solution obtained by reacting 40.00 mM glutathione with 9.98 mM selenite. The resonances are assigned for bis(glutathione)selenide (GSSeSG) and oxidized glutathione (GSSG) as indicated in the structures below:



Oxidized Glutathione, GSSG

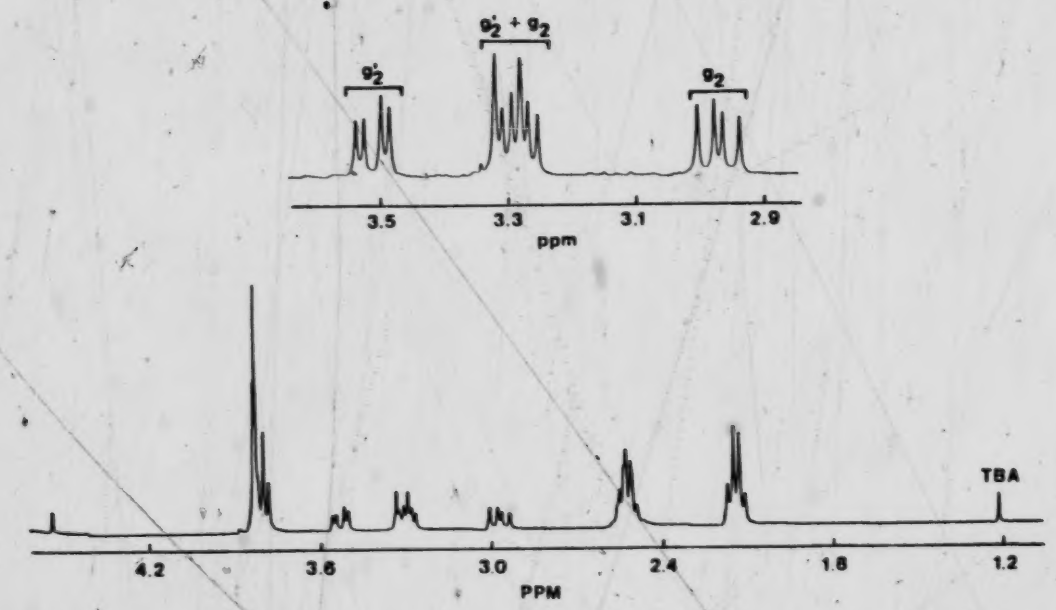
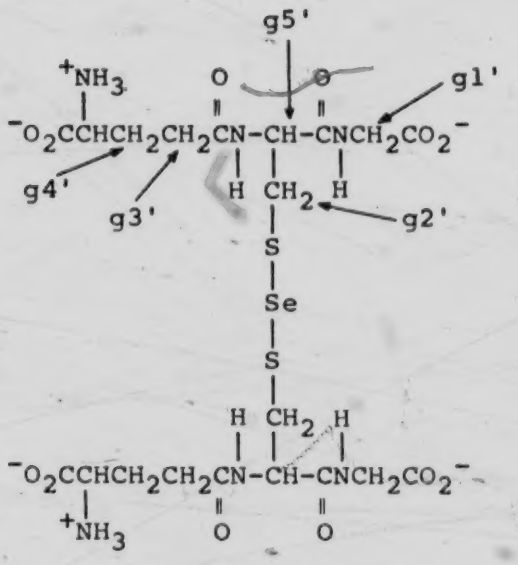


Figure 51. 360 MHz ^{1}H NMR spectrum of a mixture prepared by the reaction of 40.00 mM glutathione with 9.98 mM selenite at pH* 4.33. The inset is an expansion of the cysteinyl methylene proton region of the spectrum.



Bis(glutathione)selenide

Although the designations are labelled for one half of the structure only, they apply to both symmetrical halves. Resonances were assigned to bis(glutathione)selenide by comparing spectra of the reaction mixture with spectra obtained for authentic samples of the thiol and the disulphide. It can be clearly seen that many resonances overlap and cannot be separately resolved. For example, the glutamyl proton resonances between 2.6 ppm and 1.8 ppm are due to overlapping methylene resonances from glutathione disulphide and bis(glutathione)selenide. Some information can be gleaned from the spectral region between 2.80 ppm and 3.70 ppm, where the resonances for

the cysteinyl methylene protons are located. These resonances show spin-spin splitting typical of the AB part of an ABX spin system which is observed for the >CH-CH_2- protons in the cysteinyl residue of the peptide.

In order that the stoichiometry of the reaction of glutathione with selenite can be examined further, various reacting ratios of glutathione to selenite were studied. Figure 52 shows the cysteinyl region of ^1H NMR spectra obtained 1 minute after the start of the reaction at pH* 6.00, for thiol to selenite ratios of 2:1, 4:1, and 6:1. An interesting observation is that the spectrum for the 2:1 thiol to selenite ratio (Spectrum A in Figure 52) indicates an intermediate, having a chemical shift in the region 3.3 ppm, is formed. The intensity of this resonance was observed to decrease with time and to completely disappear 60 minutes after the start of the reaction, whereas no other obvious changes were observed elsewhere in the spectrum. At all the pH* values studied this "intermediate" resonance was observed in spectra obtained when the 2:1 thiol to selenite ratio was used. When the reaction mixture contained more than the stoichiometrically required amount of glutathione to selenite, i.e. a thiol to selenite ratio greater than 4:1, very low intensity bis(glutathione)selenide resonances were detected, as illustrated in Spectrum C in Figure

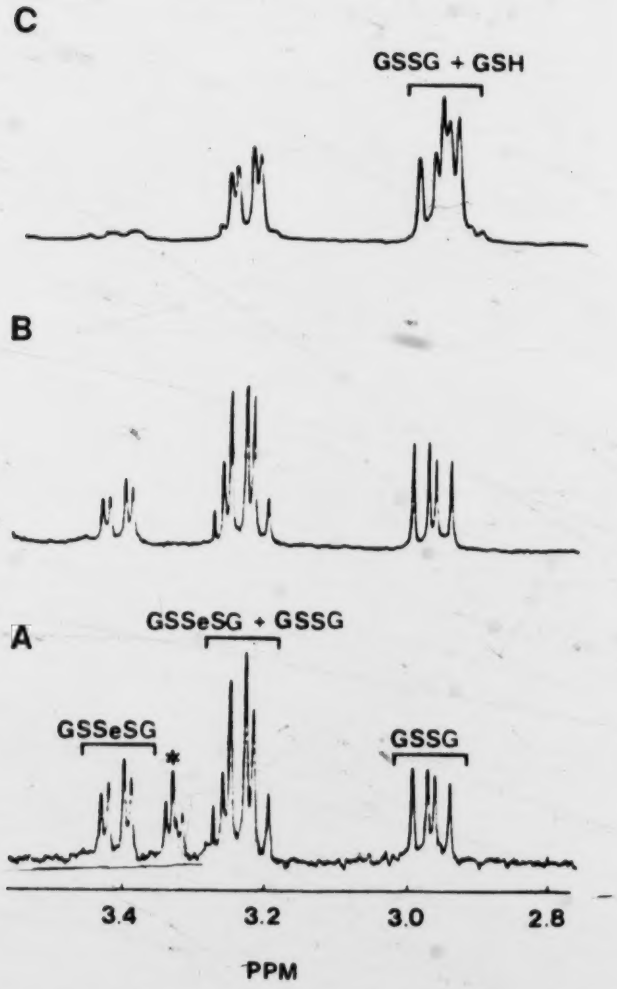


Figure 52. The cysteinyl methylene proton region of the 360 MHz ^{1}H NMR spectra of mixtures prepared by the reaction of glutathione and selenite at glutathione to selenite ratios of A: 2:1, B: 4:1, C: 6:1, at pH* 6.0.

52. The appearance of red elemental selenium indicates this is due to the rapid decomposition of bis(glutathione)selenide.

To investigate the reaction of glutathione with selenite in a 4:1 ratio by ^{1}H NMR spectroscopy, solutions were prepared with this thiol to selenite ratio over the pH* range 1.8 to 8.0. Figure 53 shows spectra obtained for the reaction of glutathione with selenite in a 4:1 ratio at various pD values. To illustrate this more closely, Figure 54 shows spectra of the cysteinyl methylene protons obtained at various times after the start of the reaction of 5.08 mM glutathione with 1.25 mM selenite at pH* 4.22. The intensities of the resonances for bis(glutathione)selenide, GSSeSG decrease with time. This is due to the decomposition of GSSeSG to the disulphide, as indicated by the appearance of red elemental selenium. At pH* 2.5, red elemental selenium was observed ~48 hours after the start of the reaction; at above pH* 4.4, this occurs in 40 minutes and above pH* 8.0, within 60 seconds. By integrating the resonances of oxidized glutathione and bis(glutathione)selenide at pH* 7.6, the rate of decomposition of the bis(glutathione)-selenide was estimated to be ~0.26 mM min^{-1}.

The complexity of these ^{1}H NMR spectra did not facilitate the characterization of the acid-base chemistry

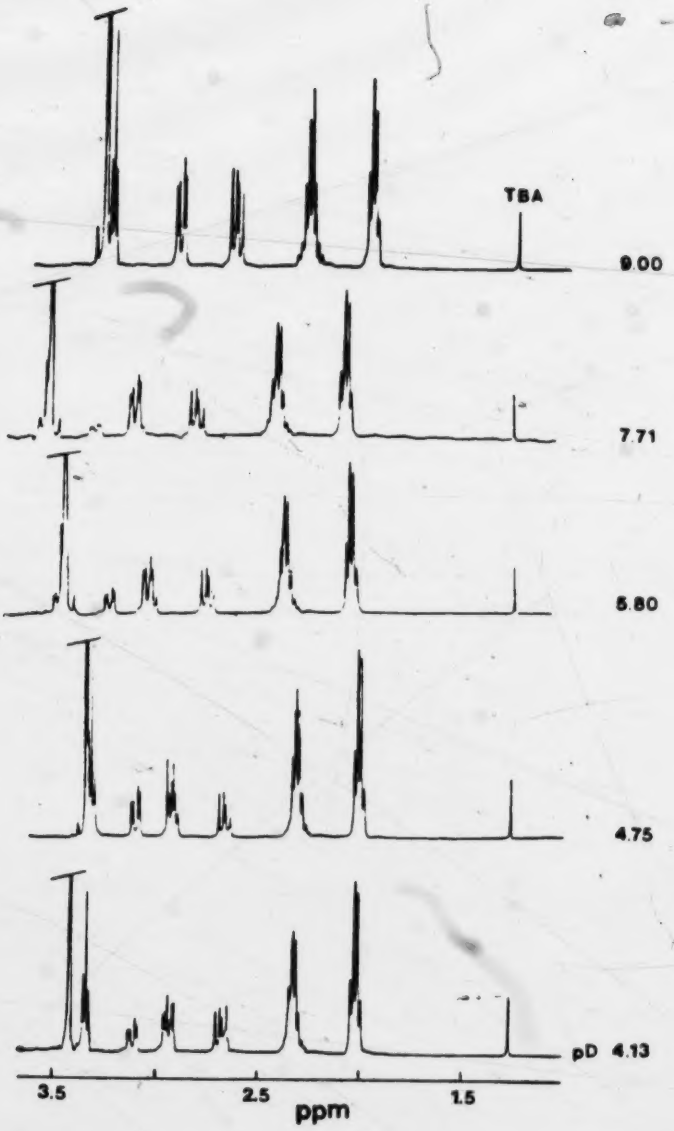


Figure 53. 360 MHz ^{1}H NMR spectra for the reaction of glutathione with selenite ($GSH:SeO_3^{2-} \cong 40$ mM:10 mM) at various pD.

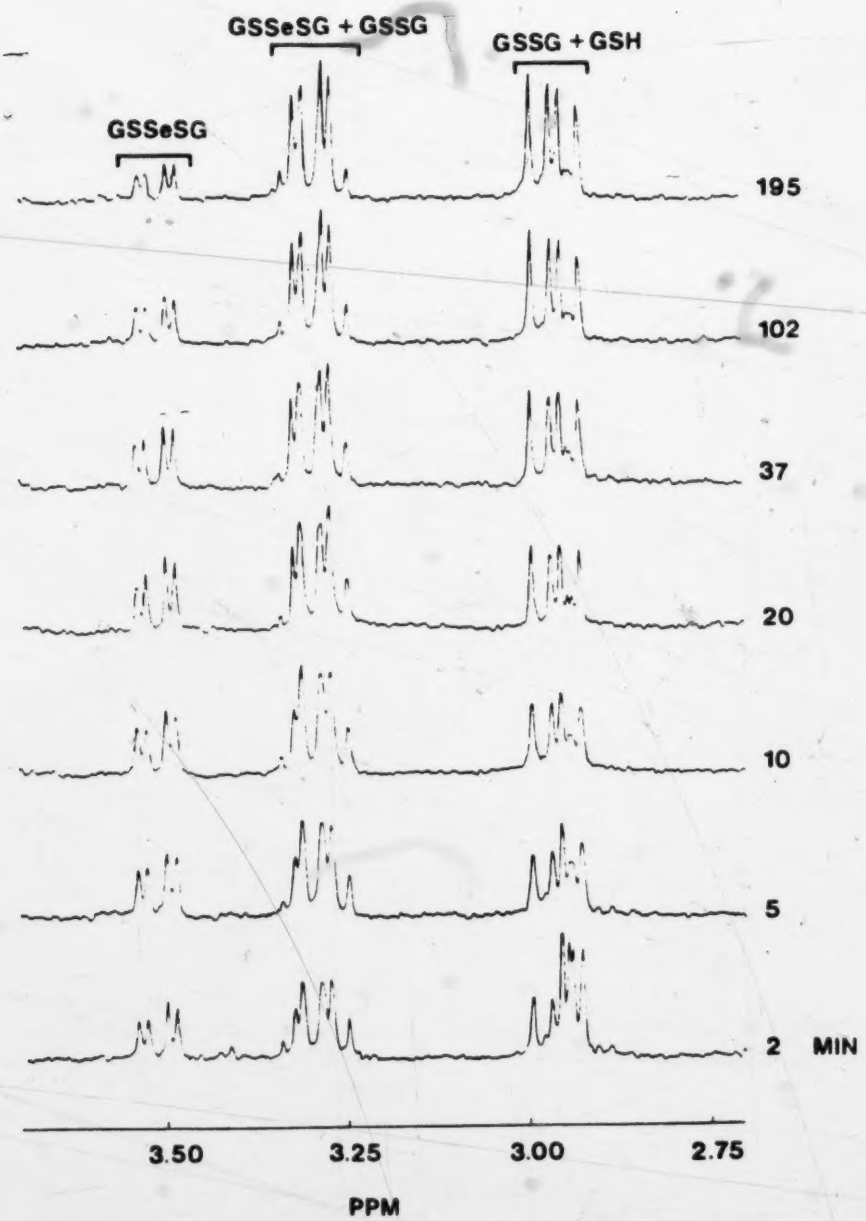


Figure 54. The cysteinyl methylene region of 360 MHz ^{1}H NMR spectra obtained during a time course study of the reaction of 5.08 mM glutathione with 1.25 mM selenite at pH* 4.22.

of bis(glutathione)selenide by ^{1}H NMR spectroscopy. The chemical shifts for the cysteinyl methylene protons for bis(glutathione)selenide and oxidized glutathione at various pD are listed in Table 20.

C. Results From ^{77}Se NMR Measurements

In the ^{1}H NMR studies, bis(alkylthio)selenides were prepared at concentrations of ~5 mM. Due to the lower sensitivity of ^{77}Se NMR, higher concentrations were required in order to obtain ^{77}Se NMR spectra within a reasonable time, i.e. less than 60 minutes. Symmetrical bis(alkylthio)selenides, RSSeSR, were prepared by adding ~0.2 M thiol solution (pH* 2) to 50 mM Na_2SeO_3 (pH* 2) at temperatures of 4 to 10°C. This often resulted in a lemon-yellow precipitate which could be re-dissolved by careful addition of dilute KOH. Asymmetrical bis(alkylthio)selenides, RSSeSR', were prepared in a similar manner except that a pH* 2 solution of a mixture of two different thiols, each at ~0.1 M, was used. All solutions were made up in 0.3 M KNO_3 for ionic strength adjustments and in 10% D_2O aqueous medium. Acidified solutions were used because the bis(alkylthio)selenides, in most cases, are unstable at alkaline pH* values, decomposing to the disulphide and red elemental selenium. In the preparation of the

Table 20. Assignment and ^1H chemical shift data for the cysteinyl methylene proton resonances in Figure 54.

pD	GSSeSG							
2.01	3.1536	3.1398	3.1137	3.0999	2.9908	2.9660	2.9469	2.9259
2.20	3.1524	3.1389	3.1125	3.0988	2.9891	2.9638	2.9470	2.9195
2.39	3.1537	3.1409	3.1133	3.1001	2.9889	2.9625	2.9485	2.9224
2.63	3.1544	3.1405	3.1138	3.1017	2.9903	2.9641	2.9505	2.9236
2.83	3.1529	3.1394	3.1128	3.0991	2.9830	2.9584	2.9434	2.9195
3.03	3.1544	3.1412	3.1145	3.1011	2.9848	2.9615	2.9452	2.9240
3.29	3.1552	3.1418	3.1152	3.1018	2.9846	2.9602	2.9425	2.9248
3.41	3.1562	3.1429	3.1163	3.1027	2.9842	2.9593	2.9441	2.9184
3.69	3.1562	3.1431	3.1162	3.1029	2.9825	2.9562	2.9425	2.9164
3.82	3.1556	3.1426	3.1156	3.1024	2.9790	2.9550	2.9392	2.9153
4.00	3.1578	3.1450	3.1175	3.1047	2.9822	2.9576	2.9418	2.9178
4.22	3.1587	3.1457	3.1186	3.1054	2.9760	2.9516	2.9358	2.9114
4.45	3.1603	3.1474	3.1204	3.1072	2.9778	2.9533	2.9376	2.9136
4.66	3.1609	3.1481	3.1209	3.1077	2.9780	2.9521	2.9383	2.9123
4.87	3.1622	3.1497	3.1217	3.1092	2.9923	2.9539	2.9397	2.9133

pD	GSSG							
2.01	2.9597	2.9465	2.9197	2.9066	2.7279	2.7024	2.6881	2.6633
2.20	2.9621	2.9472	2.9202	2.9069	2.7261	2.7003	2.6864	2.6609
2.39	2.9622	2.9485	2.9223	2.9089	2.7255	2.7000	2.6862	2.6607
2.63	2.9631	2.9499	2.9232	2.9103	2.7236	2.7007	2.6964	2.6576
2.83	2.9604	2.9492	2.9226	2.9093	2.7204	2.6943	2.6805	2.6549
3.03	2.9632	2.9516	2.9246	2.9120	2.7224	2.6964	2.6828	2.6571
3.29	2.9626	2.9530	2.9251	2.9130	2.7225	2.6967	2.6828	2.6569
3.41	2.9667	2.9542	2.9273	2.9148	2.7221	2.6962	2.6823	2.6566
3.69	2.9672	2.9549	2.9276	2.9153	2.7206	2.6945	2.6809	2.6551
3.82	2.9695	2.9563	2.9300	2.9165	2.7165	2.6904	2.6773	2.6508
4.00	2.9722	2.9588	2.9324	2.9193	2.7195	2.6936	2.6801	2.6539
4.22	2.9761	2.9628	2.9363	2.9232	2.7133	2.6871	2.6737	2.6475
4.45	2.9778	2.9645	2.9376	2.9248	2.7150	2.6887	2.6756	2.6492
4.66	2.9780	2.9656	2.9382	2.9257	2.7137	2.6873	2.6741	2.6478
4.87	2.9805	2.9678	2.9406	2.9283	2.7151	2.6887	2.6756	2.6494

bis(alkylthio)selenide solutions, a slight excess of selenite was usually used.

As no ^{77}Se NMR spectra had been reported for bis-(alkylthio)selenides the first problem was to locate ^{77}Se resonances for selenium in this form. Due to the extremely wide chemical shift range for ^{77}Se NMR, the search involved making measurements in selected regions of the spectrum. It was found in Chapter 4 of this dissertation that resonances for the alkyl selenenyl sulphides are located in the chemical shift range 250 ppm to 400 ppm. Since the selenium in bis(alkylthio)selenides is in a fairly similar chemical environment compared to the selenium in the alkyl selenenyl sulphides, it was expected that their resonances would be located close to those of the alkyl selenenyl sulphides, but to higher frequency because of their proximity to two electronegative sulphur atoms instead of one, as in the alkyl selenenyl sulphides. In the ^{77}Se NMR spectra which were obtained, this was indeed observed. The bis(alkyl-thio)selenide resonances were located in the region between 500 ppm and 700 ppm.

Spin lattice relaxation times were determined for selected symmetrical bis(alkylthio)selenides, using the inversion-recovery-pulse sequence. Figure 55 shows the ^{77}Se resonance of bis(cysteamine)selenide measured at

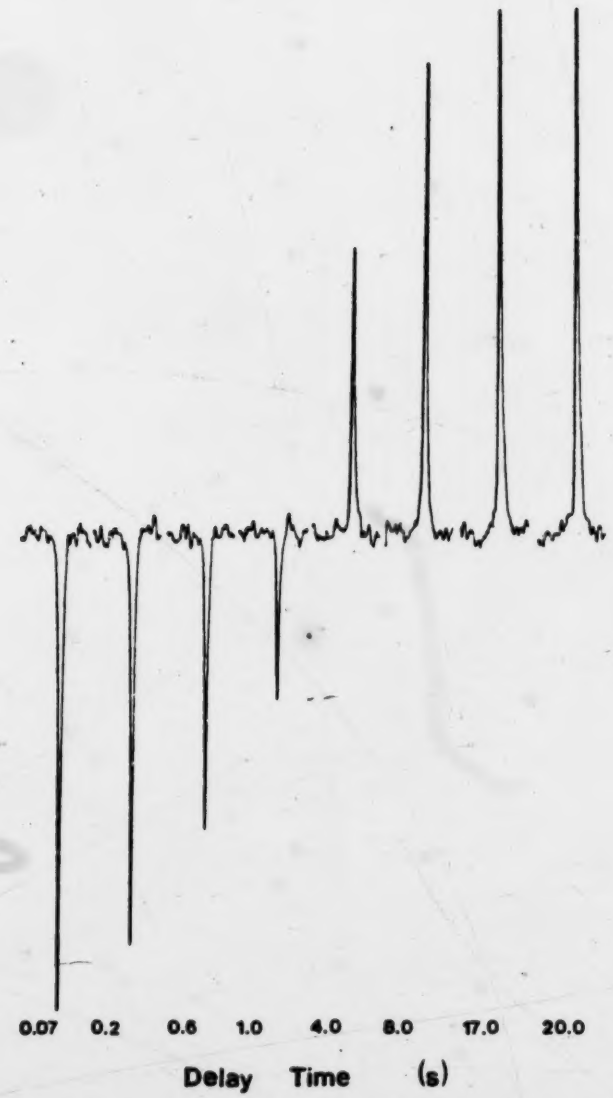


Figure 55. ^{77}Se NMR resonances of bis(cysteamine)selenide obtained in an inversion-recovery experiment to determine the ^{77}Se spin lattice relaxation time. The times given below the resonances are the delay times in the inversion-recovery-pulse sequence.

various delay times during the inversion recovery experiment. Table 21 lists the T_1 values which have been determined in this work. These results show that at millimolar concentrations the bis(alkylthio)selenides have fairly short T_1 values. The flip angle with which ^{77}Se NMR spectra can be optimally obtained was calculated using equation 77.

The ^{77}Se spectra of two symmetrical bis(alkylthio)selenides are shown in Figure 56. Figure 56A shows the ^{77}Se NMR spectrum of bis(cysteamine)selenide obtained by the reaction of 0.08 M cysteamine with 0.07 M selenite at pH* ~4. The spectrum was obtained in 30 minutes. Figure 56B shows the ^{77}Se NMR spectrum obtained for the products of the reaction of 0.2 M mercaptosuccinic acid with 0.05 M selenite at pH* 2.96. The spectrum shows a pair of ^{77}Se resonances, since a racemic mixture of the reacting thiol was used, the D-,D-(L-,L-) and D-,L-(L-,D-) bis(mercaptosuccinic acid)selenides were formed. The resonances for the stereoisomers are resolved as indicated in Figure 56B; the resonances are ~140 Hz apart. Each of these resonances is due to ~25 mM selenium and the spectrum was obtained in ~75 minutes.

Linewidths at half height for the spectra obtained for the bis(alkylthio)selenides range from 4 to 12 Hz. ^{77}Se chemical shifts for the symmetrical bis(alkylthio)-

Table 21. ^{77}Se spin lattice relaxation time of selected bis(alkylthio)-selenides.

Bis(alkylthio)selenide	T_1 (s)	pH*
Bis(cysteamine)selenide	2.69 ± 0.09	-
Bis(glutathione)selenide	0.27 ± 0.01	4.04
Bi(D-penicillamine)selenide	0.73 ± 0.06	8.72
Bis(N-acetyl penicillamine)selenide	0.70 ± 0.09	2.82
	0.87 ± 0.15	

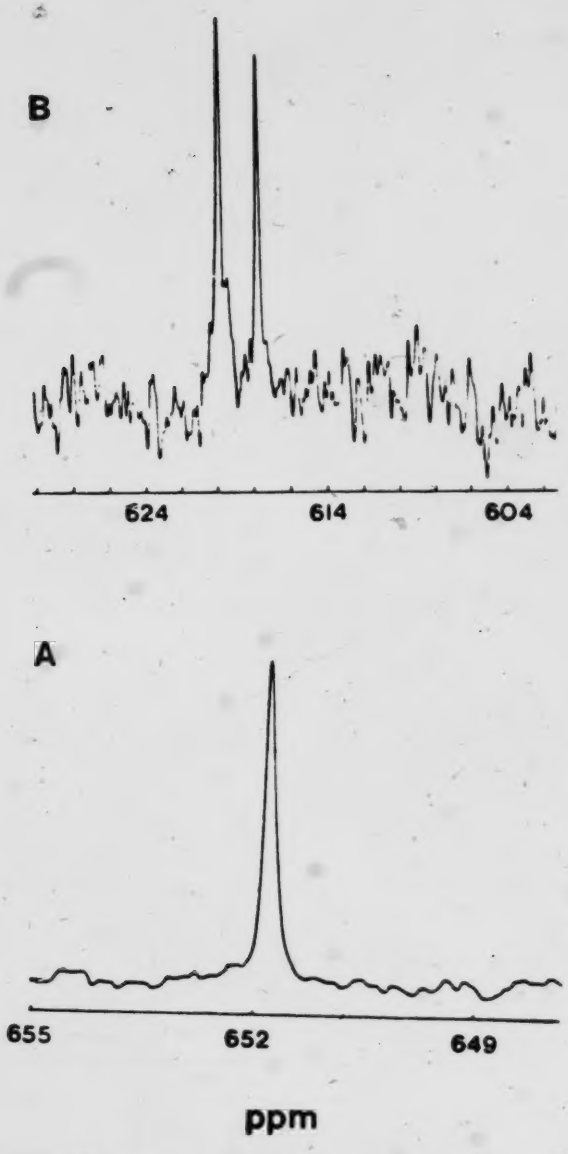


Figure 56. 68.68 MHz ^{77}Se NMR spectra of two symmetrical bis(alkylthio)selenides. Spectrum A is for bis(cysteamine)-selenide prepared by the reaction of 0.08 M cysteamine with 0.07 M selenite. Spectrum B is for bis(mercaptosuccinic acid)selenide prepared by the reaction of 0.2 M D-,L-mercaptosuccinic with 0.05 M selenite.

selenides which were studied in this work are listed in Table 22.

Figures 57-59 show ^{1}H and ^{77}Se NMR spectra of mixtures obtained by reacting selenite with two different thiols. The products for which ^{77}Se resonances are observed are the two symmetrical bis(alkylthio)selenide molecules (RSSeSR and R'SSeSR') and the asymmetrical molecule (RSSeSR'). In the cases where D-,L-enantiomers are present and/or when a complex spin-spin splitting pattern is observed, the ^{1}H NMR spectra show much overlap and resonances generally cannot be resolved for the various species in the solution mixture. The insets in the ^{1}H NMR spectra show regions from which some of the resonances can be resolved. The corresponding ^{77}Se NMR spectra, however, are relatively simple; sharp singlets are observed for all the ^{77}Se resonances detected. It is also possible to resolve resonances for stereoisomers of the asymmetrical RSSeSR' molecules.

The ^{77}Se NMR spectra obtained in Figures 57-59 show that the ^{77}Se chemical shift is very sensitive to the nature of the R and R' groups on RSSeSR'. For instance, Figure 57 is the ^{77}Se NMR spectrum of the products of the reaction of D-,L-penicillamine, D-,L-2-mercaptopropanoic acid and selenite. Six bis(alkylthio)selenides can be formed in this reaction:

Table 22. ^{77}Se chemical shifts of symmetrical bis(alkylthio)selenides.

Symmetrical Bis(alkylthio)selenides	Chemical Shift (ppm)	pH*
Bis(cysteamine)selenide	652.60	4.14
Bis(2-mercaptopropanoic acid)selenide[a]	626.56	4.21
	626.49	
Bis(D-penicillamine)selenide, Bis(L-penicillamine)selenide	551.43	4.02
D-PSSeSP-L, L-PSSeSP-D	547.71	4.02
Bis(glutathione)selenide	673.61	4.40
Bis(L-cysteine)selenide	686.74	2.41
D-CySSeSCy-L, L-CySSeSCy-D	690.68	2.95
Bis(mercaptosuccinic acid)selenide[a]	619.83	2.96
	617.70	
Bis(homocysteine)selenide[a]	647.67	7.70
	647.46	
Bis(N-acetyl penicillamine)selenide[a]	539.28	9.30
	537.06	

[a]The reaction mixture contains D-RSH and L-RSH so that D-(L-)RSSeSR-D(-L) and D-(L-)RSSeSR-L(-D) are formed; but it was not possible to assign the two resonances observed to the separate stereoisomers.

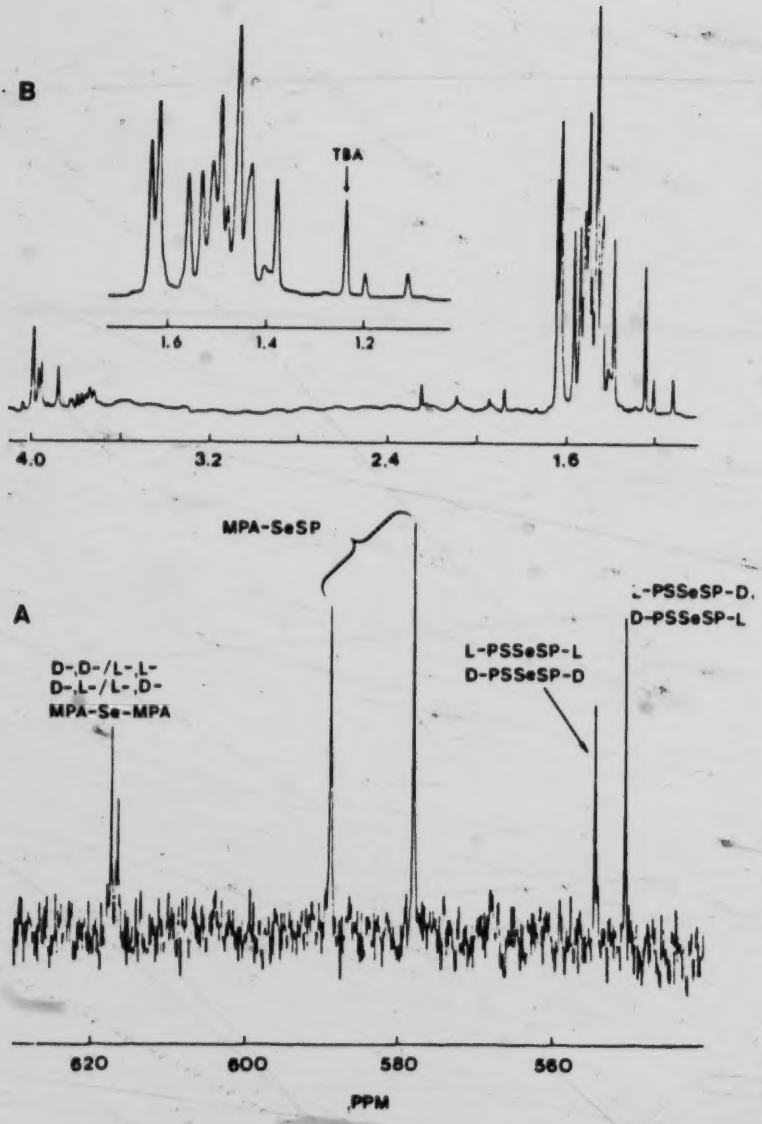


Figure 57. A: 68.68 MHz ^{77}Se NMR spectrum and B: 360 MHz ^{1}H NMR spectrum of a reaction mixture prepared by the reaction of 0.095 M 2-mercaptopropanoic acid and 0.099 M D-,L-penicillamine with 0.05 M selenite at pH* 2.27.

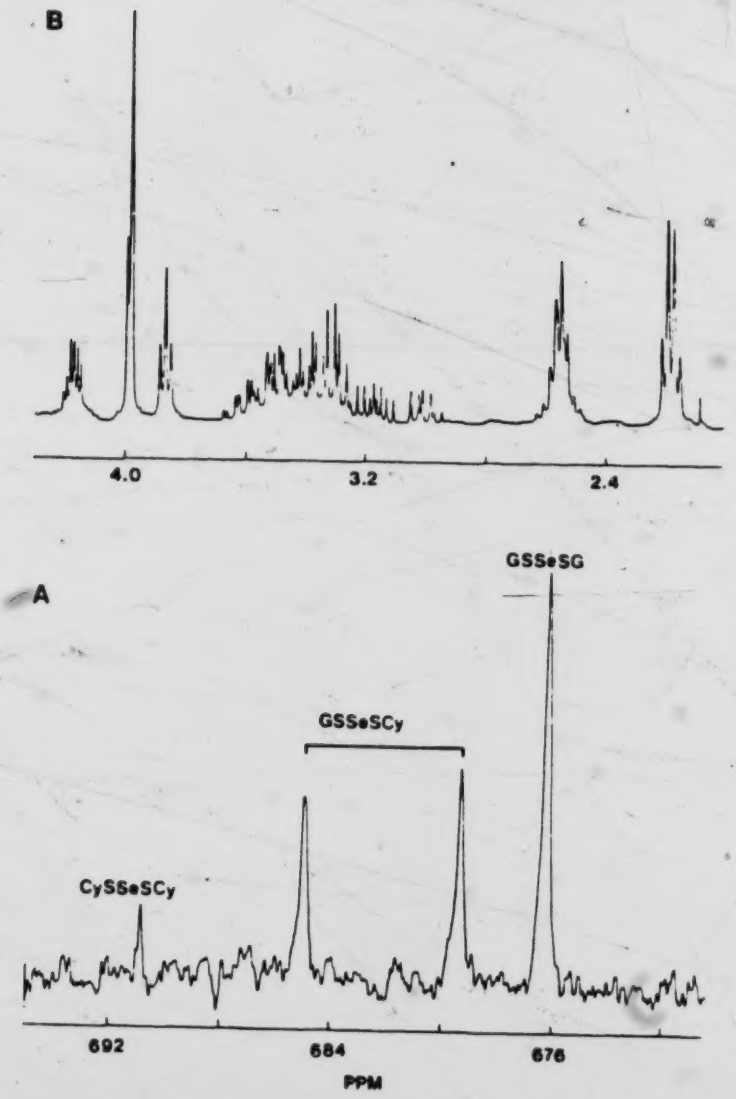


Figure 58. A: 68.68 MHz ^{77}Se NMR spectrum and B: 360 MHz ^{1}H NMR spectrum of a reaction mixture prepared by the reaction of 0.099 M glutathione and 0.095 M D-,L-cysteine with 0.05 M selenite at pH* 2.95.

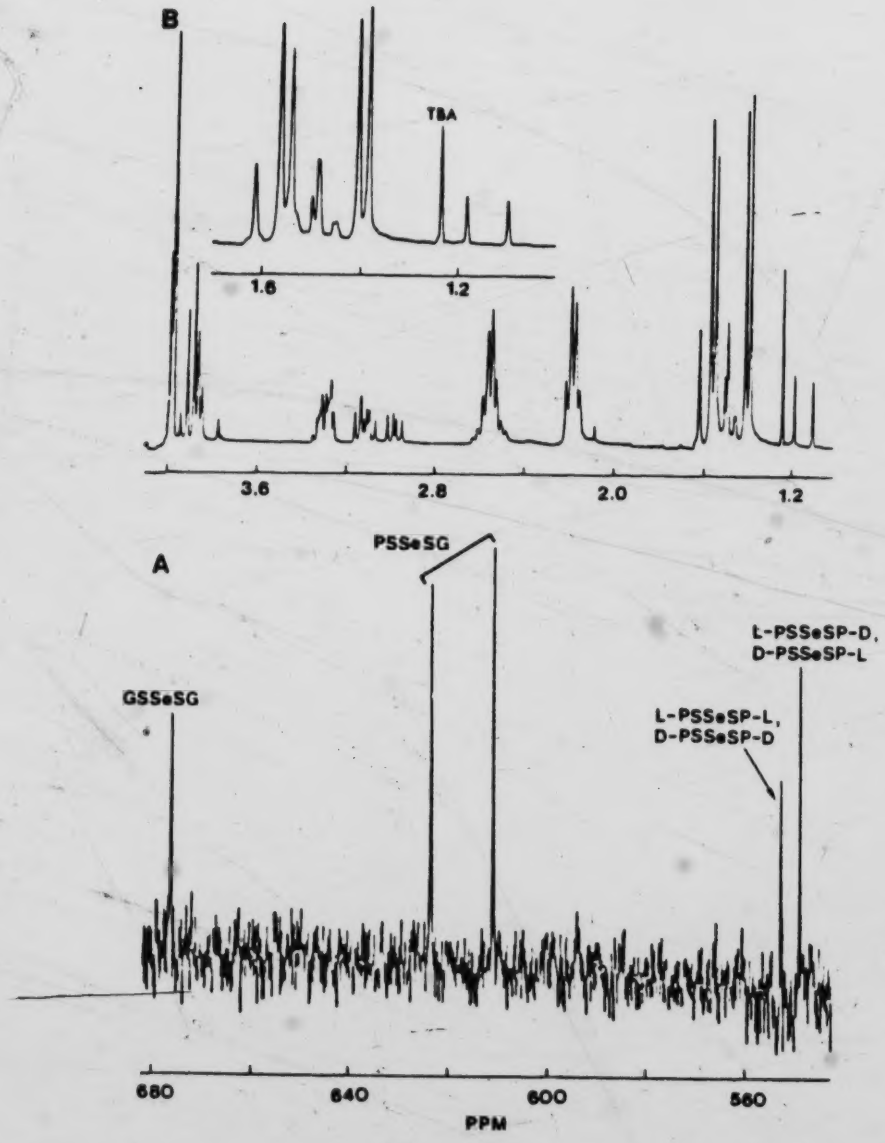
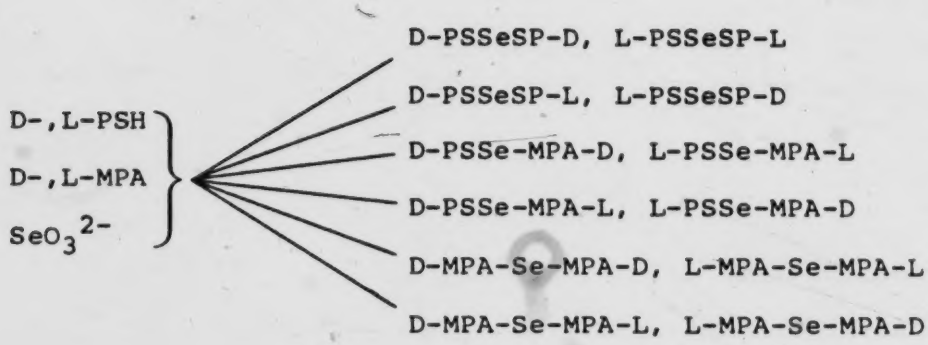


Figure 59. A: 68.68 MHz ^{77}Se NMR spectrum and B: 360 MHz ^{1}H NMR spectrum of a reaction mixture obtained by the reaction of 0.099 M glutathione and 0.099 M D-,L-penicillamine with 0.05 M selenite at pH* 2.85.



There are six resolved resonances in the ^{77}Se NMR spectrum of the reaction mixture, corresponding to the six bis-(alkylthio)selenides. Similarly, in Figures 58 and 59, all of the possible bis(alkylthio)selenides which are formed in each case can be detected in the ^{77}Se NMR spectra. This indicates that the ^{77}Se chemical shift depends on the nature of R and R' and on their conformations. Furthermore, the resonances for the asymmetrical bis(alkylthio)selenides are located between the resonances of the corresponding symmetrical bis(alkyl-thio)selenides, i.e.

$$\delta_{RSSeSR} < \delta_{RSSeSR'} < \delta_{R'SSeSR'}$$

Table 23 lists the ^{77}Se chemical shifts for the asymmetrical bis(alkylthio)selenides which have been measured in this work. The resonances have been assigned to the

Table 23. ^{77}Se chemical shifts of asymmetrical bis(alkylthio)selenides.

Asymmetrical Bis(alkylthio)selenides	Chemical Shift (ppm)	pH*
L-CySSeSP-D	619.07	2.95
D-CySSeSP-D	623.61	2.95
MPA-SeSP-D[a]	590.56	1.29
	582.80	
D-PSSeSC	602.10	3.5
D-PSSeSG	610.85	3.1
L-PSSeSG	623.35	2.90
L-CySSeSC	670.49	2.67
L-CySSeSG	684.93	2.79
D-CySSeSG	679.35	2.90
GSSeSC	666.18	2.9
D-MPASeSC, L-MPASeSC	640.85	3.88
D-MPASeSG, L-MPASeSG[a]	641.19	2.43
	649.19	
D-MPASeSCy-L, L-MPASeSCy-L[a]	652.50	2.43
	653.35	
nPSSeSP-D[a]	535.82	8.86
	536.61	
nPSSeSG[a]	606.94	4.40
	612.09	
nPSSeSCy-L[a]	610.87	3.20
	619.35	

[a]See footnote for Table 22.

respective stereoisomers by comparing spectra obtained where the reacting thiols are a racemic mixture, with those obtained when a particular stereoisomer was used. For example, the spectra shown in Figure 60 illustrate how the assignments have been made for D-PSSeSG and L-PSSeSG in spectra A and B, and D-PSSeSCy-L and L-PSSeSCy-L in spectra C and D. The results from Table 23 show that the dependence of the ^{77}Se chemical shift on R and R' is approximately additive, i.e. the chemical shift of the selenium in RSSeSR' is approximately midway between that of the selenium in RSSeSR and R'SSeSR', as illustrated by the calculations presented in Table 24. The ^{77}Se chemical shifts of the asymmetrical bis(alkylthio)selenides can be predicted accurately at any particular pH* by obtaining the average between the ^{77}Se chemical shifts of the symmetrical bis(alkylthio)selenides which are formed at the same time, if their chemical shifts are those of any specific protonated species. Otherwise, the chemical shifts of the asymmetrical molecules can be predicted to within 15 ppm from the average of the observed chemical shifts of the symmetrical molecules.

In order to investigate the use of ^{77}Se NMR to probe the solution chemistry of bis(alkylthio)selenides, the acid-base chemistry of bis(alkylthio)selenides was examined. The ^{77}Se chemical shift was monitored as a

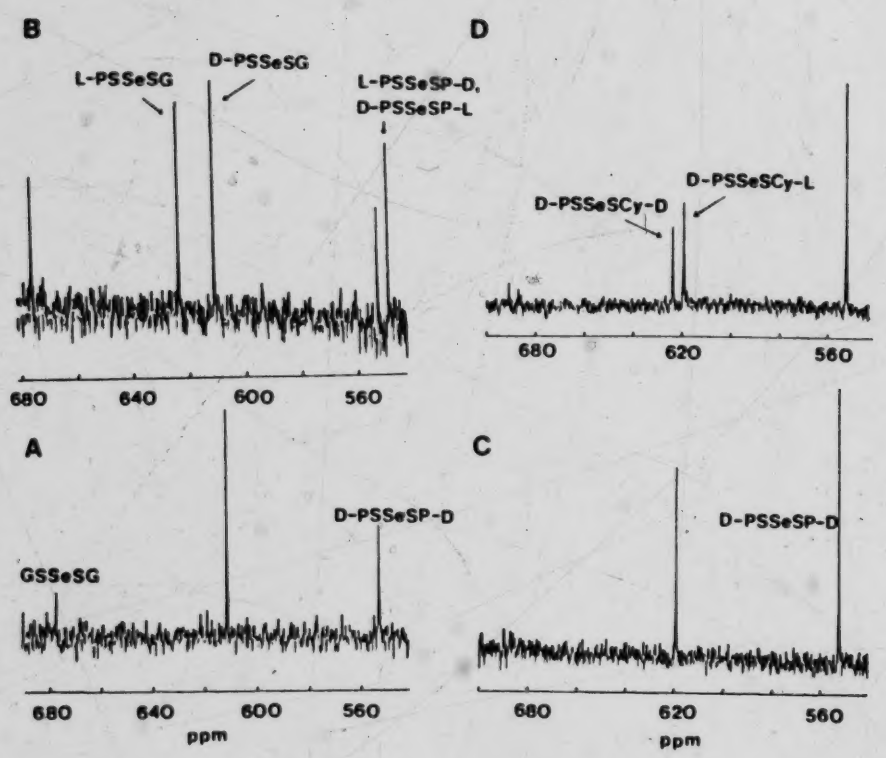


Figure 60. 68.68 MHz ^{77}Se NMR spectra for

A: a solution obtained by the reaction of 0.100 M D-penicillamine and 0.100 M glutathione with 0.05 M selenite

B: a solution obtained by the reaction of 0.099 M D-,L-penicillamine and 0.099 M glutathione with 0.05 M selenite

C: a solution obtained from the reaction of 0.099 M D-penicillamine and 0.099 M L-cysteine with 0.05 M selenite

D: a solution obtained from the reaction of 0.099 M D-penicillamine and 0.088 M D-,L-cysteine with 0.05 M selenite.

Table 24. Comparison of calculated and measured ^{77}Se chemical shifts for asymmetrical bis(alkylthio)selenides.

Symmetrical RSSeSR		Asymmetrical RSSeSR			pH*
Molecule	Chemical Shift (ppm)	Molecule (ppm)	Predicted (ppm)	Measured (ppm)	
D-PSSeSP-D	551.83				
		D-PSSeSC	602.34	602.10	3.5
CSSeSC	652.85				
CSSeSC	652.90				
		L-CySSeSC	669.92		
L-CySSeSCy-L	686.93		670.91(av.)	670.49	2.7
		D-CySSeSC	671.89		
L-CySSeSCy-D, D-CySSeSCy-L	690.88				
D-PSSeSP-D	552.03				
		L-CySSeSP-D	619.43	619.08	
L-CySSeSCy-L	686.84				2.9
		D-CySSeSP-D	621.44	623.61	
L-CySSeSCy-D, D-CySSeSCy-L	690.84				
MPA-Se-MPA[a]	617.35				
	616.45	MPA-SeSCy-L	652.03	652.55	
L-CySSeSCy-L	686.71				2.4
		MPASeSCY-L	653.68	653.47	
L-CySSeSCy-D, D-CySSeSCy-L	690.92				
GSSeSG	679.95				
		GSSeSC	666.55	666.46	1.46
CSSeSC	653.14				
L-CySSeSCy-L, D-CySSeSCy-D	686.71	L-CySSeMPA-L D-CySSeMPA-D	652.03	652.55	
L-CySSeSCy-D, D-CySSeSCy-L	690.92				
D-MPA-Se-MPA-D	617.35				2.43
L-MPA-Se-MPA-L		L-CySSeMPA-D	653.69	653.47	
D-MPA-Se-MPA-L	616.45	D-CySSeMPA-L			
L-MPA-Se-MPA-D					

[a]See footnote for Table 22.

function of pH* for selected bis(alkylthio)selenide molecules. Figure 61 shows ^{77}Se NMR spectra obtained during the titration of a solution prepared by reacting 0.099 M glutathione and 0.097 M cysteamine with 0.050 M selenite. The ^{77}Se resonance of the asymmetrical molecule, GSSeSC, is located between that of bis(glutathione)selenide, GSSeSG, to higher frequency, and bis(cysteamine)selenide, CSSeSC, to lower frequency. The ^{77}Se chemical shifts of the bis(alkylthio)selenides are plotted in Figure 62. The changes in chemical shifts are due to the titration of the carboxylic acid groups of the glutathione parts of GSSeSG and GSSeSC. The pK_A values for the carboxylic acid groups of GSSeSC were estimated from its ^{77}Se chemical shift data by curve fitting, and the results are listed in Table 25.

Because NMR spectroscopy offers the capability of studying components in a complex reaction mixture without separation, chemical shift-pH* data can be obtained not only for the asymmetrical molecules, but also for the symmetrical molecules. pK_A values estimated by non-linear least squares curve fitting for selected bis(alkylthio)-selenides are listed in Table 25. Amino pK_A values for most molecules, other than those of bis(penicillamine)-selenide could not be estimated due to the instability of bis(alkylthio)selenides above pH* 7. Also, many carboxyl

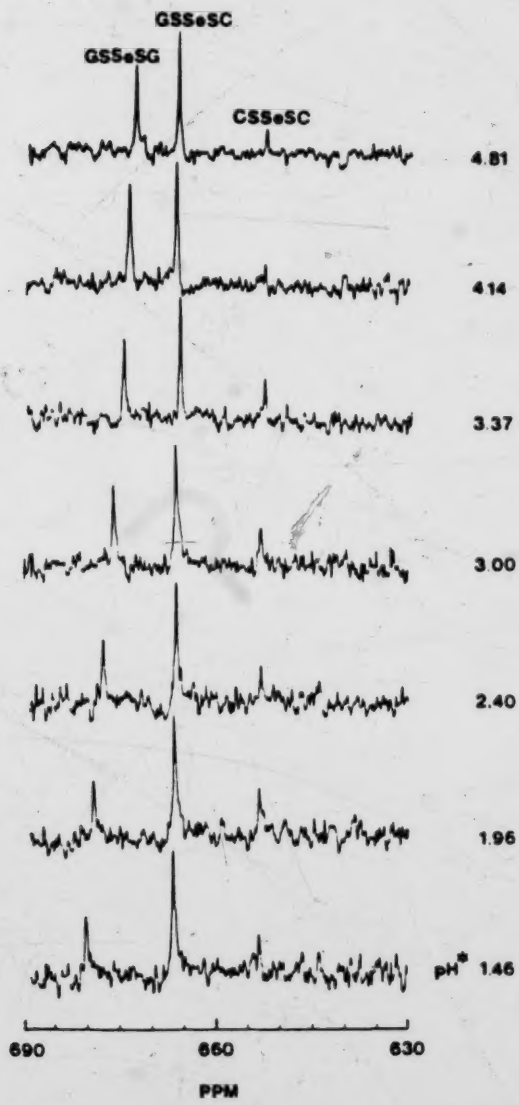


Figure 61. 68.68 MHz ^{77}Se NMR spectra obtained during the titration of the bis(alkylthio)selenides formed from the reaction of 0.099 M glutathione and 0.097 M cysteamine with 0.050 M selenite

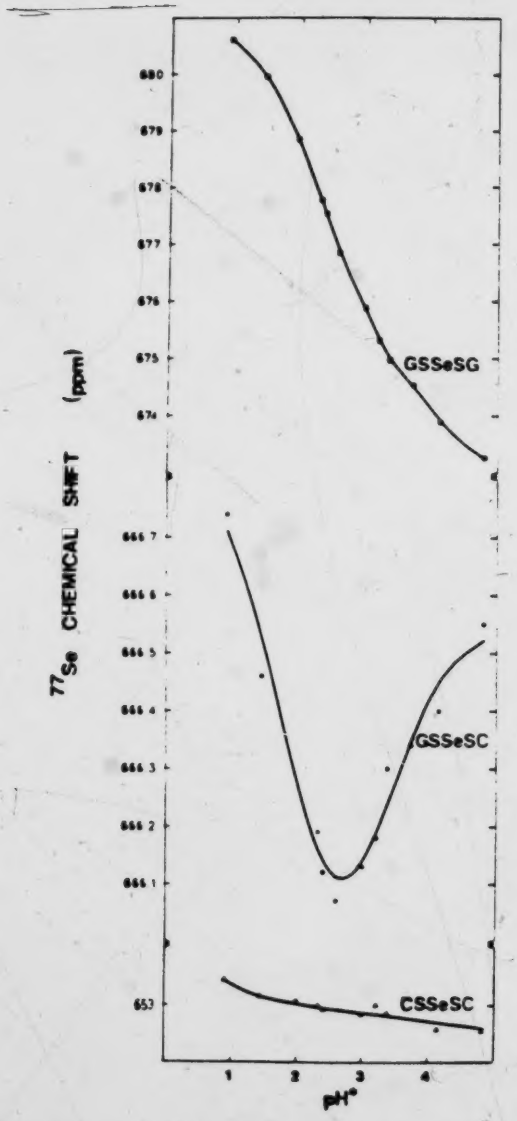


Figure 62. The pH* dependence of the chemical shift of the ^{77}Se resonances of bis(glutathione)selenide (GSSeSG), bis(cysteamine)selenide (CSSeSC) and the asymmetrical molecule, GSSeSC.

Table 25. Acid dissociation constants of selected bis(alkylthio)-selenides.

Molecule		pK_A	Limiting Chemical Shift (ppm)
D-PSSeSP-D		pK_3 = 7.71 ± 0.04	551.28 ± 0.03
		pK_4 = 8.99 ± 0.04	528.43 ± 0.05
D-PSSeSP-L, L-PSSeSP-D		pK_3 = 8.03 ± 0.04	547.58 ± 0.03
		pK_4 = 9.06 ± 0.04	527.32 ± 0.05
nPSSeSPn[a]	1.	pK_1 = 2.87 ± 0.04	545.89 ± 0.24
		pK_2 = 3.64 ± 0.05	537.73 ± 0.06
	2.	pK_1 = 2.88 ± 0.04	548.66 ± 0.17
		pK_2 = 3.69 ± 0.05	540.75 ± 0.06
GSSeSC		pK_1 = 1.76 ± 0.12	666.83 ± 0.09
		pK_2 = 3.41 ± 0.14	666.54 ± 0.05
MPA-SeSP-D[a]	1.	pK_1 = 1.84 ± 0.17	592.12 ± 0.51
		pK_2 = 3.32 ± 0.06	596.28 ± 0.09
	2.	pK_1 = 1.87 ± 0.13	584.90 ± 0.41
		pK_2 = 3.33 ± 0.07	571.32 ± 0.06

[a]See footnote for Table 22.

pK_A values could not be estimated because of changes in pK_A due to large changes in ionic strength at low pH*'s. Due to the sensitivity of the ^{77}Se nucleus to its electronic environment, the data obtained in such experiments are highly prone to media effects, such as ionic strength changes at the low and high ends of the pH scale. Estimation of acid dissociation constants from the ^{77}Se chemical shift-pH* data sets often have large uncertainty factors due to these media effects and the instability of the bis(alkylthio)selenides at alkaline pH* values.

D. Discussion

From the results which are presented in Sections B and C of this chapter, it is clear that the reaction of thiol-containing molecules with selenite in aqueous solution can be characterized by ^{1}H, ^{13}C, and ^{77}Se NMR spectroscopy. The relative intensities of the resonances for cystamine, CSSC and bis(cysteamine)selenide, CSSeSC in the ^{1}H NMR spectra of a reaction mixture prepared by reacting cysteamine, CSH with selenite, in a 4:1 ratio (see Figures 41 and 43), are approximately equal, at least at low pH* values; this is also observed in the ^{13}C NMR spectrum (Figure 42). This observation agrees well with

the results obtained by Ganther (10) according to equation (72). For the reaction of the biological thiol glutathione with selenite, this same stoichiometry is also observed at low pH*'s (Figures 51 and 54). However, as shown for 2-mercaptopropanoic acid, where the -SH group is bonded to a secondary carbon and for D-penicillamine, where the -SH group is bonded to a tertiary carbon, the ^1H NMR results indicate a different stoichiometry for the reaction of this thiol with selenite. In the case of 2-mercaptopropanoic acid, an equilibrium appears to be established. However, for penicillamine, the presence of additional unassigned resonances indicates the formation of other reaction products at pH* values below 6.0; the results also indicate that bis(penicillamine)selenide is being formed in larger quantities than penicillamine disulphide, contrary to that predicted by equation (72). An equilibrium situation is also observed for the reaction of penicillamine with selenite, which can be characterized if the additional products are identified. The proposed 4:1 stoichiometric reaction is observed for 2-mercapto-propanoic acid and penicillamine at neutral pH* values up to ~8.0, which is the upper pH* limit in this study. ^1H NMR spectra from time course studies suggest that it may be possible to characterize the kinetics of the reaction of penicillamine with selenite at pH* values below 6.0.

In the studies by both Ganther (10-12) and Kice (14), the reaction of thiols with selenite is reported to yield only one selenium-containing product. The observation of only one ^{77}Se resonance for the symmetrical non-stereoisomeric bis(alkylthio)selenides formed by the reaction of the alkyl thiol with selenite is in agreement with these reports. ^{77}Se NMR is by far a more superior technique for establishing the presence of a selenium-containing product because it is not necessary to decompose the compound for analysis of the reaction products. This suggests that the use of ^{77}Se NMR spectroscopy can therefore be an excellent probe for conducting investigations on the incorporation of selenium into protein, since the selenium atom can be monitored directly and non-destructively.

Ganther (10) noted that the reaction of cysteine with selenite was sensitive to the thiol to selenium ratio. For instance, bis(cysteine)selenide showed a marked tendency to decompose at ratios higher than 4:1, and if the reaction was carried out using high concentrations of the reactants at room temperature. It was also observed in this work that bis(cysteine)selenide is less stable compared to the other bis(alkylthio)selenides, as extensive decomposition had occurred at the end of the experiment time for acquiring a ^{77}Se NMR spectrum. The

^{77}Se NMR spectrum shows a resonance with low intensity and signal-to-noise ratio, although the concentrations used were comparable to those used for other thiols. The stability of bis(penicillamine)selenide up to pH 7.0 which was noted earlier (85), is confirmed in this study; in this work, a solution containing bis(penicillamine)selenide can be obtained at alkaline pH values up to 12 without any decomposition to penicillamine disulphide and red elemental selenium, if the high pH is not maintained for long periods.

From ^{1}H NMR results on symmetrical bis(alkylthio)selenides it has been shown that although a pure sample of the compound is not available, its acid-base chemistry can be characterized by monitoring the variation of the chemical shift of these resonances with pH; the acid dissociation constants of the protonated groups on these molecules can be estimated, as detailed in Chapter 2.

^{77}Se NMR studies have shown that only one selenium-containing product is formed (in the absence of isomeric species of the thiol) in detectable quantities, in the reaction of thiols with selenite. This technique of measurement has also been shown to be useful for the characterization of the solution chemistry of molecules which have complex ^{1}H NMR spectra because ^{77}Se resonances

are sharp singlets under the conditions used in these studies. The sensitivity of the ^{77}Se chemical shift to the nature and conformation of the R and R' groups on the bis(alkylthio)selenides is demonstrated in the ability to detect separate resolved resonances for stereoisomeric species. Hence, it should be a very useful probe to employ for monitoring the selenium nucleus.

From ^{77}Se chemical shift results presented in Tables 22 and 23, the following additional observations can be made:

1. R groups on symmetrical bis(alkylthio)selenides which contain carboxylic acid groups as the only functional groups with dissociable protons are located between 610 ppm and 630 ppm, as exemplified by the bis(alkylthio)selenides of 2-mercaptopropanoic acid and mercaptosuccinic acid. The ^{77}Se chemical shifts for bis(2-mercaptopropanoic acid)selenide and bis(mercaptosuccinic acid)selenide are given below at comparable pH*'s:

RSSeSR	Chemical Shift (ppm)	pH*
Bis(2-mercaptopropanoic acid)selenide	618.90	3.00
	619.54	
Bis(mercaptosuccinic acid)selenide	617.70	2.96
	619.83	
Bis(penicillamine)selenide	533.2	9.30
	531.4	
Bis(N-acetylpenicillamine)selenide	537.06	9.30
	539.28	

From this chemical shift data, the substitution of a methyl proton with a carboxyl group does not appear to cause significant shift to high frequency in the ^{77}Se chemical shifts of the resonances. Also listed are the ^{77}Se chemical shifts of the stereoisomeric species for bis(penicillamine)selenide and bis(N-acetylpenicillamine)-selenide at pH* 9.30. The effect of acetylation of the amino group appears to be a shift to higher frequency of ~6 ppm at this pH*, although the selenium atom is located at a distance of four bonds from the acetylation site.

2. The effect of substituting a methyl group for a proton is reflected in the shift to lower frequency of the ^{77}Se resonances. This can be better appreciated by comparing the chemical shifts of several molecules as shown below:

Bis(alkylthio)selenide	pH*	Chemical Shift (ppm)	Difference (ppm)
D-PSSeSG	2.80	611.95	72.98
L-CySSeSG	2.79	684.93	
D-PSSeSCy-L	2.95	619.07	71.61
D-CySSeSCy-L	2.95	690.68	
D-PSSeSP-D	2.97	552.74	70.87
D-CySSeSP-D	2.95	623.61	
D-PSSeSC	3.5	602.10	68.39
L-CySSeSC	2.67	670.49	
D-PSSeSP-D, L-PSSeSP-L	2.80	553.4	133.4
L-CySSESCy-L, D-CySSeSCy-D	2.79	686.80	
D-PSSeSP-L, L-PSSeSP-D	2.80	549.8	140.88
L-CySSeSCy-D, D-CySSeSCy-L	2.95	690.68	

The difference in the chemical shift due to this substitution is ~70 ppm to lower frequency. This effect would be expected to double when the number of methyl groups doubles; this is indeed observed in the symmetrical bis(alkylthio)selenides, bis(penicillamine)selenide and bis(cysteine)selenide.

3. The ^{77}Se chemical shifts of symmetrical bis(alkylthio)selenides formed by the reaction of cysteine and glutathione with selenite show that there is a shift

to lower frequency when R in RSSeSR is a peptide compared to an amino acid. ^{77}Se chemical shift data which reflect this are shown below.

Bis(alkylthio)selenide	pH*	^{77}Se Chemical Shift (ppm)	Difference (ppm)
D-PSSeSCy-L	2.95	619.07	7.82
D-PSSeSG	3.04	611.25	
L-CySSeSC	2.67	670.49	4.31
GSSeSC	2.60	666.07	
L-CySSeSCy-L	2.79	686.80	10.39
GSSeSG	2.79	676.41	
MPA-SeSCy-L	2.43	652.50 653.35	11.31 4.16
MPA-SeSG	2.43	641.19 649.19	

The chemical shift differences vary from 4 ppm to 11 ppm due to the different R groups, and it is not possible to suggest a representative value for the chemical shift difference.

A comparison of the amino pK_A values for bis(D-penicillamine)selenide and D-,D-penicillamine disulphide in D_2O shows that the presence of selenium in these otherwise identical molecules does not result in significant differences in the acid dissociation constants

for the amino groups:

D-,D-Penicillamine Disulphide	Bis(D-Penicillamine)selenide
1. $pK^D_3 = 8.34 \pm 0.03$	1. $pK^D_3 = 8.24 \pm 0.04$
$pK^D_4 = 9.29 \pm 0.04$	$pK^D_4 = 9.56 \pm 0.02$
2. $pK^D_3 = 8.40 \pm 0.04$	2. $pK^D_3 = 8.32 \pm 0.02$
$pK^D_4 = 9.40 \pm 0.04$	$pK^D_4 = 9.48 \pm 0.05$

The difference between the average pK^D_3 and pK^D_4 values obtained for D-,D-penicillamine disulphide is 0.98 pK units, while for bis(D-penicillamine)selenide it is 1.24 pK units. The presence of a selenium atom between two sulphur atoms appears to cause a slight increase in the pK^D_A values estimated for these molecules.

In Chapter 4, ^{77}Se NMR has been shown to be suitable for characterizing the acid-base chemistry of diselenides. Results from both ^1H and ^{77}Se NMR measurements on bis(D-penicillamine)selenide are shown in Figure 63. The amino pK_A values obtained for the chemical shift-pH* data are:

1. ^{77}Se NMR: $pK_3 = 7.71 \pm 0.03$
   $pK_4 = 8.87 \pm 0.03$
2. ^1H NMR: $pK_3 = 7.84 \pm 0.04$
   $pK_4 = 8.98 \pm 0.03$

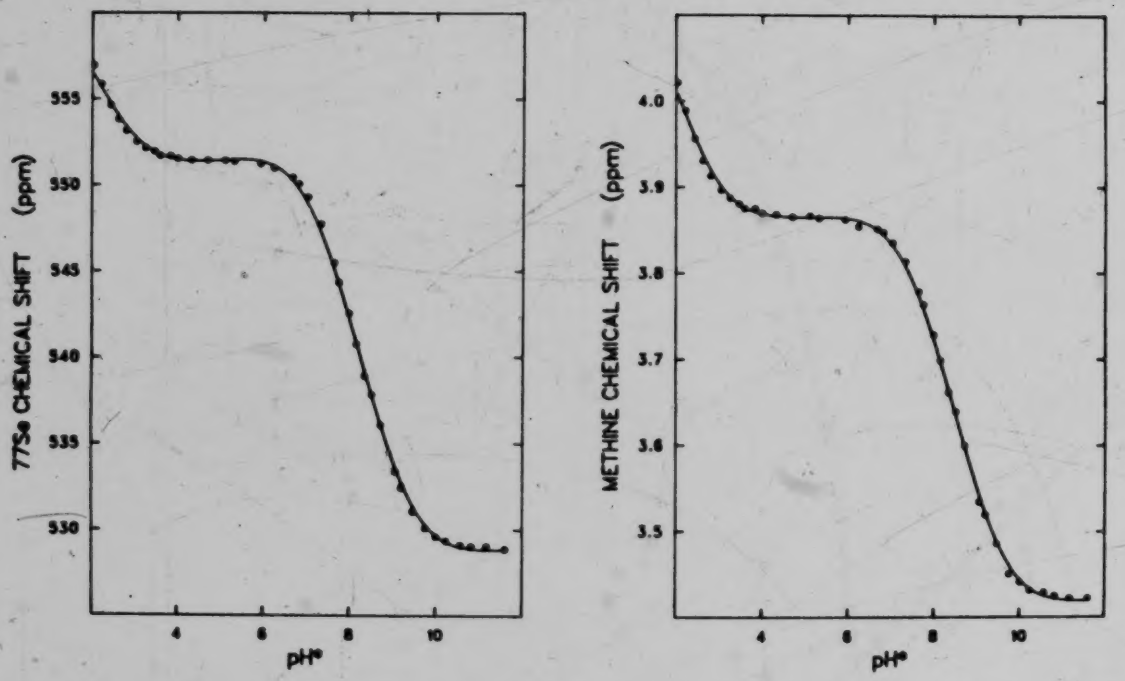


Figure 63. NMR titration curves for a solution prepared by reacting 0.099 M D-penicillamine with 0.025 M Na_2SeO_3. A: ^{77}Se chemical shift and B: ^{1}H methine proton chemical shift of bis(D-penicillamine)selenide is plotted as a function of pH*.

The average values determined for these acid dissociation constants are 7.78 and 8.93, respectively. Hence ^{77}Se NMR is again shown to be capable of yielding results with comparable accuracy when used to probe the solution chemistry of selenium-containing compounds.

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APPENDIX A

The computer subroutine program which was written for use with the non-linear least squares curve-fitting program KINET is listed below. The parameter which is to be determined is the displacement constant, K_d from which formation constants may be calculated, as derived in Chapter 3. The subroutine has been written for a selenol having 3 dissociable protons and is duly altered for monoprotic and diprotic selenol molecules.

```
 1      C THIS SUBROUTINE CALCULATES THE EQUILIBRIUM
          CONSTANT, KD, FOR:
 2      C MEHGSR + RSE- = MEHGSER + RS-
 3      C BY FITTING THE OBSERVED CHEM.SHIFT OF COMPETING
          RS-.
 4
 5      C THE SELENOL HAS 3 DISSOCIABLE PROTONS
 6
 7            SUBROUTINE EQN
 8            IMPLICIT REAL*8(A-H,K,O-Z)
 9            COMMON
               ZZZZZ,III,I999,ITAPE,I998,JTAPE,I997,IWT,
               I996,LAP,
10          C I994,XINCR,NOPT,I993,NOVAR,I992,NOUNK,I991,
               X,U,ITMAX,I990,
11          C WTX,TEST,I,I889,AV,RESID,IAR,I888,EPS,ITYP,
               I887,XX,RXTYP,
12          C DX1I,FOP,FO,FU,P,ZL,TO,EIGVAL,XST,T,DT,L,
               I886,M,I885,JJJ,
13          C I884,Y,DY,VECT,NCST,I880,CONST
14            DIMENSION X(5,200),U(20),WTX(5,200),XX(5),
               FOP(200),FO(200),
15          C FU(200),P(20,21),VECT(20,21),ZL(200),TO(20),
               EIGVAL(20),
16          C XST(200),Y(10),DY(10),CONST(20)
17            GOTO(2,3,4,5,1),ITYP
18       1    CONTINUE
19            ITAPE=5
```

```
20            JTAPE=6
21      C     INSERT NOUNK AND NOVAR ASSIGNMENTS HERE
22            NOUNK=1
23            NOVAR=3
24            RETURN
25       2    CONTINUE
26
27      C PKA'S OF LIGANDS AND MEHG-LIGAND SPECIES
28            KAMS=10.**(-CONST(1))
29            KA1MSE=10.**(-CONST(2))
30            KA2MSE=10.**(-CONST(3))
31            KA1RS=10.**(-CONST(4))
32            KA2RS=10.**(-CONST(5))
33            KA1RSE=10.**(-CONST(6))
34            KA2RSE=10.**(-CONST(7))
35            KA3RSE=10.**(-CONST(8))
36      C EXPERIMENTAL OBSERVABLES
37            H-10.**(-XX(1))
38            DELOBS=XX(2)
39            VOL=XX(3)
40      C TOTAL COMPONENT CONCENTRATIONS (CORRECTED FOR
          DILUTION)
41            VINIT=CONST(17)
42            DILFAC=VINIT/(VINIT+VOL)
43
44            HGTOT=CONST(9)*DILFAC
45            STOT=CONST(10)*DILFAC
46            SETOT=CONST(11)*DIFLAC
47      C CHEMICAL SHIFTS OF RS-CONTAINING SPECIES
48            DRS=CONST(12)
49            DRSH=CONST(13)
50            DRSH2=CONST(14)
51            DRSM=CONST(15)
52            DRSMH=CONST(16)
53      C DISPLACEMENT EQUILIBRIUM CONSTANT (NOT
          CONDITIONAL)
54            KD=10.**(U(1))
55      C SET UP EQUATIONS IN RSTOT
56
57      C CALCULATE ALPHA'S FOR PROTONATED SPECIES
              AMS=1+H/KAMS
59            AMSE=1+H/KA2MSE+H*H/KA1MSE/KA2MSE
60            ARS=1+H/KA2RS+H*H/KA1RS/KA2RS
61            ARSE=1+H/KA3RSE+H*H/KA2RSE/KA3RSE+H*H*H/
              KA1RSE/KA2RSE/KA3RSE
62      C EXPRESS CONDITIONAL FORMATION CONSTANTS IN TERMS
          OF ALPHA'S
63            KCD=KD*ARS*AMSE/AMS/ARSE
64
65            A=KCD-1
66            B=KCD*(HGTOT-SETOT-2*STOT)-HGTOT+STOT
```

```
      C=KCD*STOT*(STOT+SETOT-HGTOT)

C SOLVE THE QUADRATIC AZ2+BZ+C=0
      DET=B*B-4*A*C
      IF(DET.LT.0.0) STOP
      R1=(-B+DSQRT(DET))/2/A
      R2=(-B-DSQRT(DET))/2/A
      IF(R1.LE.0.0.AND.R2.LE.0.0) STOP
      IF(R1.GE.STOT.AND.R2.GE.STOT) STOP
      IF((R1.GT.0.AND.R1.LT.STOT).AND.(R2.GT.0.AND
     .R2.LT.STOT)) STOP
      IF(R1.GE.STOT.OR.R1.LE.0.0) RSTOT=R2
      IF(R2.GE.STOT.OR.R2.LE.0.0) RSTOT=R1

C EXPRESS OTHER RS-CONTAINING SPECIES IN TERMS OF Z
      RS=RSTOT/ARS
      RSH=RS*H/KA2RS
      RSH2=RS*H/KA1RS

      RSMTOT=STOT-RSTOT
      RSM=RSMTOT/AMS
      RSMH-RSM*H/KAMS

      DELC=(DRS*RS+DRSH*RSH+DRSH2*RSH2+DRSM*RSM+
     DRSMH*RSMH)/STOT
      RESID=DELC-DELOBS
      RETURN

    3 CONTINUE
    4 CONTINUE
    5 CONTINUE
      RETURN
      END
```